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Abstract

This thesis explores the potential of Algerian-sourced beetroot (*Beta vulgaris*), supplemented with additives or combined with carob pods and soybeans, as a sustainable alternative to conventional Man, Rogosa, and Sharpe (MRS) agar for cultivating Lactic Acid Bacteria (LAB). This study investigates the performance of these formulated plant-based media in supporting the growth of *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Enterococcus durans*, focusing on growth rate, time of generation, acid production and productivity. Various formulations, including Beetroot Juice with functional additives like Manganese sulfate (MS) and ammonium citrate (AC) were tested, along with carob-beetroot and soybean-beetroot blends. Results showed that beetroot juice alone was insufficient for bacterial growth, but mineral supplementation significantly improved microbial performance. For instance, the carob-beetroot blend (CBB) yielded μ_{max} values of 0.251 h^{-1} for *L. plantarum* and 0.275 h^{-1} for *L. fermentum*, along with an acid production rate (Q_{ac}) of $4.051 \text{ g/g}\cdot\text{h}$ for *L. fermentum*. Additionally, the soybean-beetroot blend (SBB) supported *L. plantarum* growth with a μ_{max} of 0.248 h^{-1} and a generation time of 2.79 h, while maintaining consistent acid production ($Q_{ac} = 0.161 \text{ g/g}\cdot\text{h}$). In comparison, MRS displayed a μ_{max} of 0.154 h^{-1} and higher acid production ($Q_{ac} = 1.588 \text{ g/g}\cdot\text{h}$). These findings suggest that plant-based beetroot media could replace MRS in LAB cultivation, providing a sustainable, cost-effective, and biodegradable alternative for fermentation processes in food biotechnology.

Keywords: Beetroot, Carob, Soybean, Lactic acid bacteria, fermentation, growth medium

الملخص

تستعرض هذه الأطروحة إمكانيات الشمندر السكري المستورد من الجزائر (*Beta vulgaris*)، المدعم بالإضافة أو المدموج مع قرون الخروب وفول الصويا، كبدائل مستدام لأجار مان، وروغوسا، وشارب (MRS) التقليدي لزراعة بكتيريا حمض اللاكتيك (LAB). تتناول هذه الدراسة أداء الوسائط النباتية المُشكلة في دعم نمو بكتيريا *Lactobacillus plantarum*، *Lactobacillus fermentum*، *Enterococcus durans*، مع التركيز على معدل النمو، ووقت التوليد، وإنتاج الحمض، والإنتاجية. تم اختبار عدة تركيبات، بما في ذلك عصير الشمندر مع إضافات وظيفية مثل كبريتات المنغنيز (MS) وسيتريت الأمونيوم (AC)، بالإضافة إلى خلطات الشمندر والخروب وخلطات الشمندر وفول الصويا. أظهرت النتائج أن عصير الشمندر وحده كان غير كافٍ لنمو البكتيريا، لكن الإضافات المعدنية حسّنت بشكل كبير أداء الكائنات الدقيقة. على سبيل المثال، حققت خليط الشمندر والخروب (CBB) قيم μ_{max} قدرها 0.251 ساعة⁻¹ لـ *L. plantarum* و 0.275 ساعة⁻¹ لـ *L. fermentum*، بالإضافة إلى معدل إنتاج الحمض (Qac) قدره 4.051 جرام/جرام·ساعة لـ *L. fermentum*. علاوة على ذلك، دعم خليط الشمندر وفول الصويا (SBB) نمو *L. plantarum* بقيمة μ_{max} قدرها 0.248 ساعة⁻¹ ووقت توليد قدره 2.79 ساعة، مع الحفاظ على إنتاج الحمض بشكل ثابت (Qac = 0.161 جرام/جرام·ساعة). بالمقارنة، أظهرت MRS قيمة μ_{max} قدرها 0.154 ساعة⁻¹ وإنتاج حمض أعلى (Qac = 1.588 جرام/جرام·ساعة). تشير هذه النتائج إلى أن الوسائط النباتية القائمة على الشمندر يمكن أن تحل محل MRS في زراعة بكتيريا حمض اللاكتيك، مما يوفر بديلاً مستداماً وفعالاً من حيث التكلفة وقابل للتحلل لعمليات التخمير في تكنولوجيا الغذاء الحيوية.

الكلمات الرئيسية: البنجر، الخروب، فول الصويا، بكتيريا حمض اللبنيك، التخمير، وسط النمو

Résumé

Cette thèse explore le potentiel de la betterave d'origine algérienne (*Beta vulgaris*), supplémentée avec des additifs ou combinée avec des gousses de caroube et du soja, en tant qu'alternative durable à l'agar Man, Rogosa et Sharpe (MRS) pour la culture des bactéries lactiques (LAB). Cette étude examine les performances de ces milieux d'origine végétale formulés dans le soutien à la croissance de *Lactobacillus plantarum*, *Lactobacillus fermentum* et *Enterococcus durans*, en se concentrant sur le taux de croissance, le temps de génération, la production d'acide et la productivité. Différentes formulations, y compris le jus de betterave avec des additifs fonctionnels comme le sulfate de manganèse (MS) et le citrate d'ammonium (AC), ont été testées, ainsi que des mélanges caroube-betterave et soja-betterave. Les résultats ont montré que le jus de betterave seul était insuffisant pour la croissance bactérienne, mais que l'ajout de minéraux améliorerait considérablement les performances microbiennes. Par exemple, le mélange caroube-betterave (CBB) a donné des valeurs de μ_{\max} de $0,251 \text{ h}^{-1}$ pour *L. plantarum* et de $0,275 \text{ h}^{-1}$ pour *L. fermentum*, ainsi qu'un taux de production d'acide (Q_{ac}) de $4,051 \text{ g/g}\cdot\text{h}$ pour *L. fermentum*. De plus, le mélange soja-betterave (SBB) a soutenu la croissance de *L. plantarum* avec un μ_{\max} de $0,248 \text{ h}^{-1}$ et un temps de génération de $2,79 \text{ h}$, tout en maintenant une production d'acide constante ($Q_{\text{ac}} = 0,161 \text{ g/g}\cdot\text{h}$). En comparaison, MRS a montré un μ_{\max} de $0,154 \text{ h}^{-1}$ et une production d'acide plus élevée ($Q_{\text{ac}} = 1,588 \text{ g/g}\cdot\text{h}$). Ces résultats suggèrent que les milieux végétaux à base de betterave pourraient remplacer MRS dans la culture des LAB, offrant ainsi une alternative durable, économique et biodégradable pour les processus de fermentation en biotechnologie alimentaire.

Mots-clés : Betterave, Caroube, Soja, Milieu de culture, Bactéries lactiques, Fermentation, .

List of publications

1-**Oussama, B.k**, Fatima, S., Ahmed, H. et al. Formulations of novel microbial medium from beetroot powder: a sustainable substitute for commercial Man-de-Rogosa Sharp agar (MRS) in cultivating lactic acid bacteria. **World J Microbiol Biotechnol** 41, 372 (2025). <https://doi.org/10.1007/s11274-025-04561-x>

2-**Kamel B O**, Sahnouni F, Hariri Ahmed H. Greener Cultivation: Replacing Man Rogosa and Sharpe Agar with Beetroot and Soybean Formulated Medium for Lactic Acid Bacteria Cultivation. *Trop J Nat Prod Res.* 2025; 9(9): 4484 – 4494 <https://doi.org/10.26538/tjnpr/v9i9.49>

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List of Abbreviations

MRS: de Man, Rogosa, and Sharpe Medium

BJFA: Beetroot Juice with Fixed Additives

BJFA-MS: Beetroot Juice with Fixed Additives + Magnesium Sulfate

BJFA-SM: Beetroot Juice with Fixed Additives + Manganese Sulfate

BJFA-CA: Beetroot Juice with Fixed Additives Citrate ammonium

BJFA-AS: Beetroot Juice with Fixed Additives Acetate de Sodium

BJFA-PP: Beetroot Juice with Fixed Additives Phosphate di potatium

BJFA-ZO: Beetroot Juice with Fixed Additives Zink Oxid

CFU (UFC) – Colony Forming Units (Unités Formant Colonies)

CBB: Carob–Beetroot Blend

BJFA-T80: Beetroot Juice with Fixed Additives Tween 80

SBB: Soybean Beetroot Blend

DO: Optical Density

GC-MS : Gas Chromatography–Mass Spectrometry

HPLC : High-Performance Liquid Chromatography

LAB : Lactic Acid Bacteria

MIC : Minimum Inhibitory Concentration

PCR : Polymerase Chain Reaction

pH : Potential of Hydrogen (measure of acidity)

qX : Specific Growth-Associated Biomass Rate

SGR (μ) : Specific Growth Rate

G : Time of Generation (doubling time)

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Introduction

General Introduction

The cultural fabric of Western Algeria is interwoven with the traditional application of medicinal plants, which form an integral part of spiritual customs, healthcare systems, and community practices. This botanical knowledge transfers across generations through oral tradition. Local nutritionist employ species such as *Beta vulgaris* and *Ceratonia siliqua* along as functional food for treating diverse health conditions, with these plants carrying both healing properties and bioactive compound importance. Even with the expansion of contemporary medical practices, medicinal plant usage continues to thrive, especially in remote regions where access to standard healthcare facilities is constrained (Mahmoud et al., 2022). The generational transfer of this traditional knowledge maintains the relevance of these natural remedies, establishing them as essential healthcare resources for numerous communities.

Simultaneously, employing biotechnological approaches to valorize these plants is essential for identifying novel bioactive compounds, thus benefiting both regional and international markets (Zatout et al., 2021). For example, Red beetroot (*Beta vulgaris* L.), a reddish tuberous stem vegetable widely consumed globally, exists in several varieties with colors ranging from yellow to red, with the deep red variety being the most cultivated and utilized (Ibraheem et al., 2016; Lee et al., 2014). Additionally, the carob tree (*Ceratonia siliqua* L.), an evergreen species belonging to the Fabaceae family and Caesalpiniaceae subfamily, is highly drought-tolerant, allowing it to thrive in harsh environments. This resilience contributes to its productivity, and carob trees are widely cultivated across numerous countries (Hadi et al., 2017). Soy (*Glycine max*), a leguminous crop native to East Asia, holds a significant place in global agriculture due to its high protein content, adaptability, and versatile applications. Soybean cultivation has expanded across various climate zones worldwide, with approximately 85% of the crop being grown in the United States, Brazil, and Argentina. Soybeans are a primary source of plant-based protein and healthy fats, and they also find numerous uses beyond food production, including adhesives, plastics, biofuels, and compostable materials, underlining their relevance to environmentally conscious markets (Junaidi et al., 2025; L. Liu et al., 2022; Rizzo, 2024).

The pursuit of sustainable, environmentally conscious alternatives across diverse biotechnological sectors has generated enthusiasm for investigating plant-derived formulations for cultivating lactic acid bacteria (LAB). LAB species, including *Lactobacillus* genus, and *Enterococcus* genus, serve critical functions in multiple biotechnological applications, notably in food fermentation and probiotic manufacturing. These microorganisms, celebrated for their positive impacts on human wellness, conventionally proliferate in synthetic culture media such as de Man, Rogosa, and Sharpe (MRS). While effective, MRS medium presents environmental and financial limitations stemming from its dependence on synthetic components (Sawicki et al., 2024). This situation has prompted investigation into alternative cultivation media that are simultaneously sustainable, economically viable, and capable of rivaling the performance of traditional synthetic options. Recent years have witnessed growing attention toward plant-derived

media, which present multiple benefits including natural accessibility, minimized environmental footprint, and economic efficiency (O'Connor et al., 2015; Sahin, 2017). Plants contain abundant sugars, polyphenolic compounds, antioxidants, and dietary fiber, positioning them as promising candidates for LAB cultivation substrates. Nevertheless, despite their favorable characteristics, plant-based media face constraints related to their inherent composition and nutritional content.

1.1. Research Gap: Requirement for Sustainable Culture Media

Although plant-derived media have demonstrated certain potential, they have not yet achieved the efficacy of MRS medium, particularly regarding optimal LAB growth support. Current research indicates that while plants like beetroot can facilitate LAB multiplication, their nutritional composition frequently proves inadequate, requiring supplementation with additional components (Adekolurejo et al., 2023). This study confronts the significant gap in developing a cost-effective, plant-based medium capable of adequately supporting LAB growth while replicating the advantages of conventional synthetic media. Since the medium that targeted this gap are scarce and we cite the one from (Malik et al., 2019a) who conducted research on the fermentation of beetroot juice, carrot juice, and an equal mixture of both for 72 hours by the following LAB: *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus acidophilus*. Their findings exposed that LAB were viable at concentrations greater than 6 Log CFU/ml, which complies with the probiotic juice standards for storage, and the sensory properties were also maintained. Moving Forth our challenge involved engineering a formulation that maintains equilibrium among essential nutrients required for LAB development, frequently demanding synergistic interactions between diverse plant materials. By investigating a combination of beetroot, carob, and soybean, this research seeks to optimize the medium and address the shortcomings of single-plant formulations.

- **Beetroot (*Beta vulgaris*)**, constitutes a nutrient-dense plant serving as the foundation of this investigation. Acknowledged for its substantial content of vitamins, minerals, and antioxidants, beetroot is especially appreciated for its betalains, phenolic constituents, and fiber, which confer numerous health advantages (Malik et al., 2019a; Ninfali & Angelino, 2013a).
- **Carob (*Ceratonia siliqua*)**, a leguminous tree indigenous to Mediterranean regions, contributes to this medium through its elevated polyphenol concentration, recognized for facilitating microbial proliferation (Ait Ouahioune et al., 2022).
- **Soybean (*Glycine max*)**, valued for its high protein and amino acid concentration, additionally augments the medium's nutritional profile. Soybean's capability to supply essential growth factors including amino acids and proteins proves critical for satisfying the metabolic requirements of LAB (Ayu et al., 2023; Wolf et al., 1983).

This research employs a methodical approach to engineer a plant-based medium for LAB cultivation. In this investigation, three plant-derived media underwent development and evaluation

for their capacity to support the growth of the following LAB: *L. plantarum*, *L. fermentum* and *E. durans*.

- **Initial Phase:** assessed the proliferation of three lactic acid bacteria strains in beetroot juice (BJ) as the exclusive medium. Subsequently, beetroot juice received supplementation with additional nutrients, encompassing yeast and meat extract, Tween 80 and peptone. Further experimental trials then examined the incorporation of components including magnesium sulfate, ammonium citrate, and dipotassium phosphate into the juice, evaluating their influence on bacterial development relative to the fundamental BJ medium and comparing results with the standard MRS medium.
- **Second Phase:** Following beetroot-based medium assessment, the formulation incorporated two selected plants—carob and soybean. These plants aim to complement the nutritional components in beetroot juice by refining the medium composition. Specifically, the Carob-Beetroot Blend (CBB) and Soybean-Beetroot Blend (SBB) were formulated to achieve an improved and more comprehensive composition appropriate for Lactic Acid Bacteria.

The primary target of this research is to contribute to the development of sustainable microbiological practices by formulating an substitute growth media for lactic acid bacteria (LAB) through the valorization of locally available plant resources. Specifically, the study is guided by the following objectives:

- To investigate the nutritional potential of selected plant-based substrates,
- To formulate and evaluate a novel semi-natural growth medium

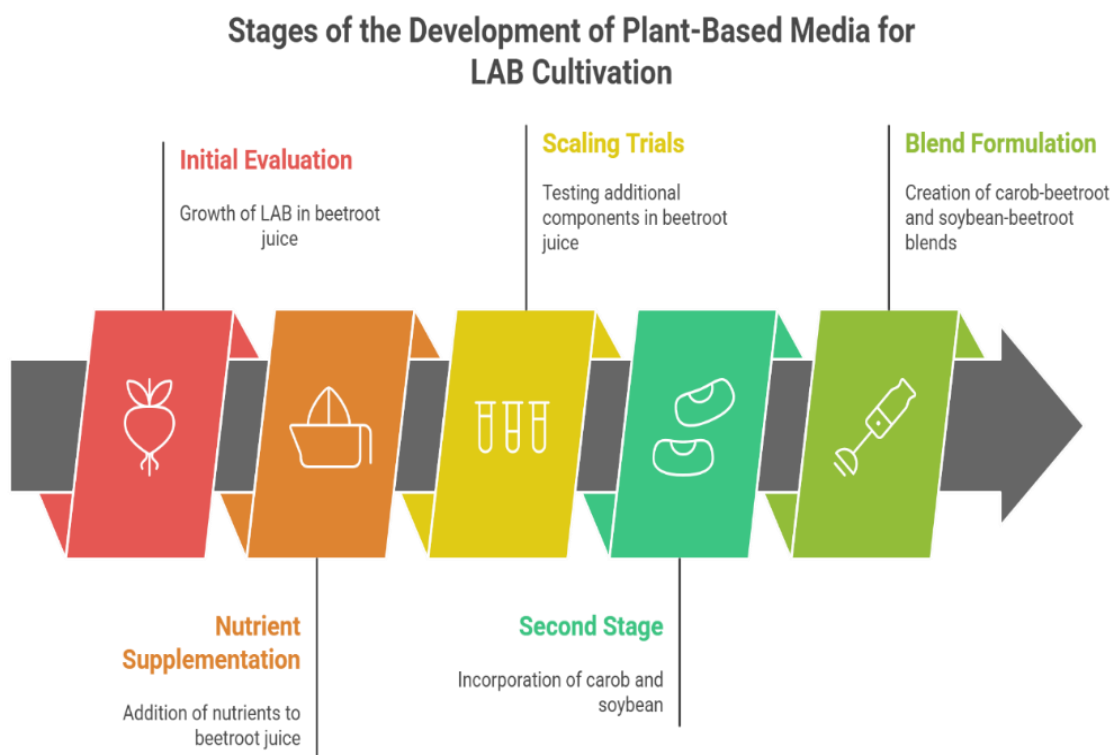


Figure 1. Illustration of the stages of research

1.2. This study is guided by several key hypotheses:

- Plant-derived nutrients, when combined appropriately, can support and enhance LAB growth, making plant-based media a viable alternative to synthetic formulations.
- The combination of beetroot, carob, and soybean will create a synergistic effect that improves bacterial growth performance compared to individual plant-based sources.
- The plant-based medium formulated from these ingredients will be nutritionally comparable to MRS medium, providing equivalent support for LAB growth while being more cost-effective and environmentally sustainable.

Part 1 Literature Review

Chapter 1

Biotechnological and
health benefits of Beetroot
(Beta vulgaris L.)

1. Beetroot: Chemical Profile and Biotechnological Potential

1.1. Introduction to Beetroot's

Red beetroot (*Beta vulgaris* L.) a reddish tuberous stem vegetable commonly consumed around the world, comes in several varieties with colors ranging from yellow to red, with the deep red variety being the most widely cultivated and used (Ibraheem et al., 2016; Lee et al., 2014). The anatomical structure of beetroot comprises the roots (hypocotyl), the sprout (which consists of semi-rigid red to purple stems, also referred to as petioles), and the leaves (leafy limbs), which are tender and vary in color and shape depending on the beet cultivar (Kumar, 2015; Ninfali and Angelino, 2013b). Beetroot counts among the *Amaranthaceae* family which is distinguishable by elevated quantities of betalains, polyphenols, and various phytochemical components that are linked to antioxidant activities (Georgiev et al., 2010; Lalonde & Roitberg, 1992). Due to the elevated amount of sugar and anti-oxidative stress control, beets are largely favoured as a reservoir of dietary sugar and Studies rank beets among the 10 most potent vegetables concerning antioxidant content (Fissore et al., 2013; Frank et al., 2005; Kurowska & Manthey, 2002; Vinson et al., 1998).

Conversely, the available body of literature has showcased that beetroot possesses unique nutritional value and contains a plethora of bioactive compounds (de Oliveira et al., 2021). Despite this fact, few studies focus on beetroot as a carrier for the probiotic attribute. This review aims to focus on their value as a functional food and the biotechnological beneficial attributes that they possess.

1.2. Botanical classification

- Kingdom: *Plantae*
- Phylum: *Streptophyta*
- Class: *Equisetopsida*
- Subclass: *Magnoliidae*
- Order: *Caryophyllales*
- Family: *Amaranthaceae*
- Genus: *Beta*
- Species: *Beta vulgaris*
- According to : (*Achparaki, et al., 2012*)



Figure 2. The beetroot plant aspect

1.2.1. Geographical distribution according to (POWO, 2025)

Beetroot (*Beta vulgaris*) is indigenous to some areas where it has been cultivated for millennia. Over the time it has been introduced to numerous other countries. Its widespread cultivation in temperate regions worldwide is primarily due to human agricultural activities. For a clearer understanding of its distribution, (figure 3).

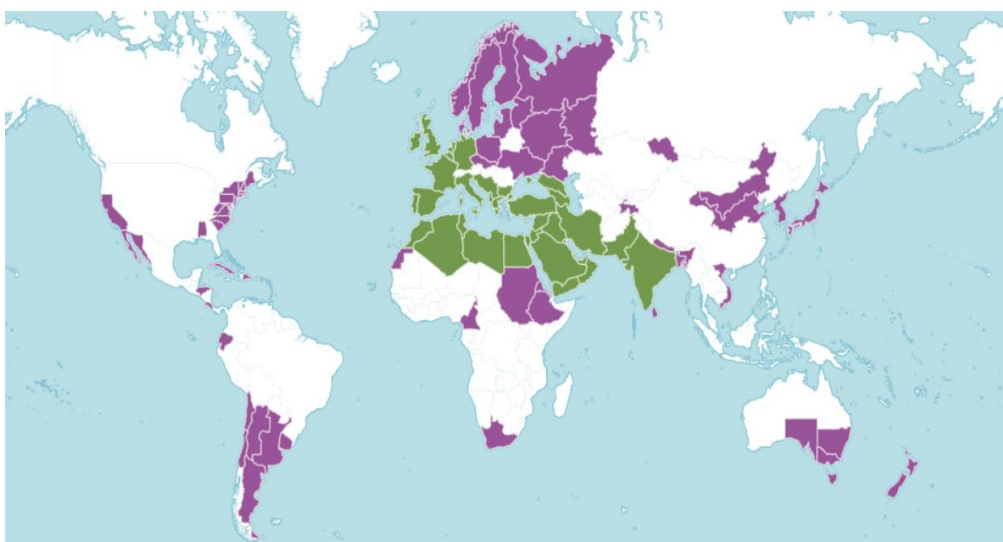


Figure 3. Distribution of *Beta vulgaris* worldwide wide Green: Native. Purple: introduced

1.3. Beetroot Cultivation Regions in Algeria

Beetroot (*Beta vulgaris*) is cultivated in multiple regions of Algeria, particularly in areas with suitable climatic conditions and soil types. Notable regions include:

1. **Adrar:** situated in the southern regions of Algeria.
2. **Ouargla:** Situated in central regions of the country.
3. **El Oued:** Located in the southeastern part of Algeria.
4. **Ghardaïa:** Ghardaïa is included in the national plan for beet cultivation.
5. **Biskra:** Located in northeastern Algeria



Figure 4. A map highlights the five key regions in Algeria known for beetroot cultivation: Adrar, Ouargla, El Oued, Ghardaïa, and Biskra

1.4. Chemical and Nutritional Profile in *Beta Vulgaris*

Beetroot has been noted to contain valuable source of bioactive chemicals including fiber, and multiple minerals counting: potassium, sodium, iron, copper, magnesium, calcium, phosphorus and Zinc, as for the vitamins it was recorded to hold : retinol, ascorbic acid, and B-complex. The most heavily researched phytochemicals in beetroot are betalains, along with some phenolic compounds. Beetroot possesses high nutritional value due to its high sugar content, mainly as bioavailable sucrose (Lundberg et al., 2008; van Velzen et al., 2008). Based on the data reported, A 100 mL serving of beetroot juice contains an average of 95 Kcal and 43 kcal of energy, along with 22.6 g of carbohydrates, 0.70 g of protein, 0.16 g of total lipids, and between 2.8 g and 0.91 g of total dietary fiber, and 12 g of total sugars. As such, sugar fraction of that 100 mL beetroot juice is around: 8.8 g sucrose, 0.86 g fructose, and 2.5 g glucose (Baião et al., 2017; Nayik, 2020; USDA, 2012).

Beetroot is a powerhouse of nutrients, including essential vitamins like folate, which is detrimental for cell division and the formation of red blood cells. In addition, beetroot is a good reservoir of potassium and iron, which support heart function and oxygen transport in the blood. The fiber content in beetroot is beneficial for gastrointestinal health, helping to regulate bowel movements and prevent constipation. Furthermore, beetroot's high nitrate content is associated with improved cardiovascular health, as it helps to lower blood pressure by enhancing nitric oxide levels in the blood vessels (Thiruvengadam et al., 2024) see Figure 5.

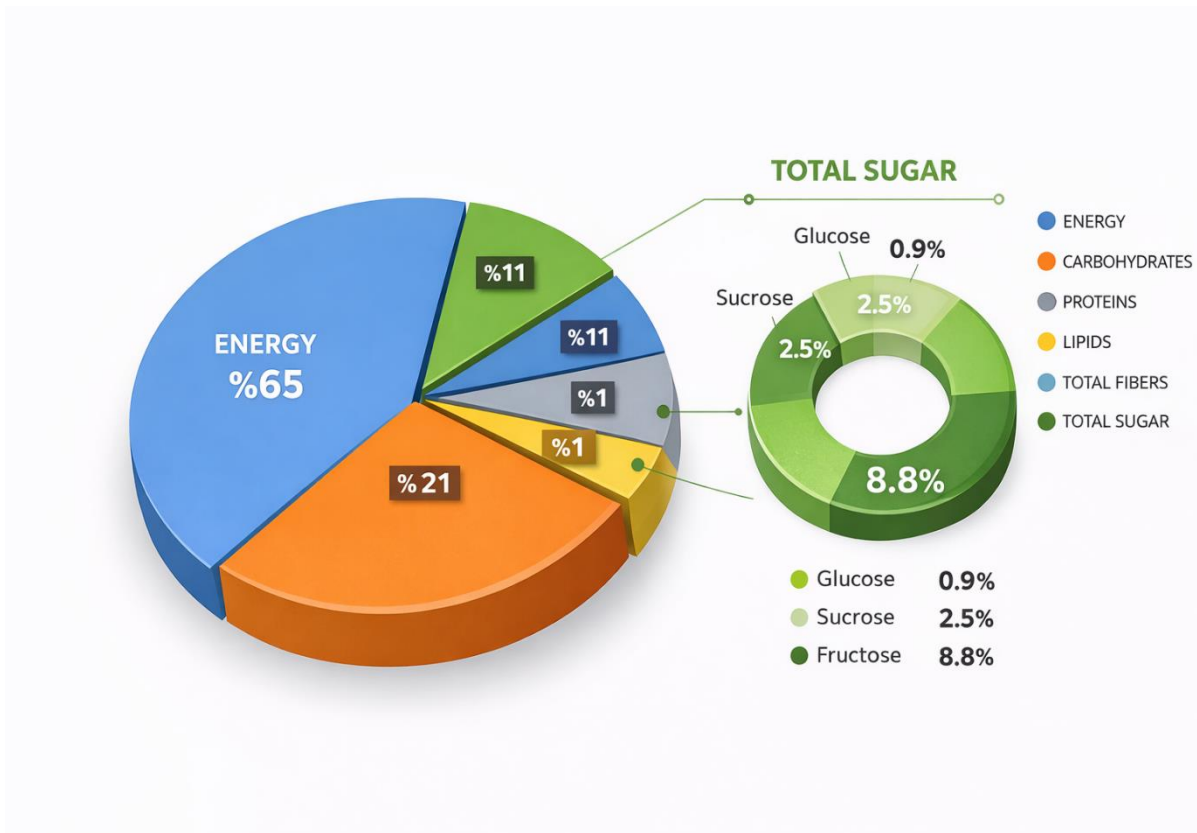


Figure 5. Composition of 100 ml of beetroot

related to the works of (Baião et al., 2017; L. Chen et al., 2021b; Nayik, 2020; USDA, 2012)

Table 1. Beetroot nutritional and chemical composition in 100 g

	Nutriments	Raw beetroot
Macro-nutriments	Water	87.58 mg
	Energy	43 kcal
	Protein	1.61 gm
	Carbohydrate	9.56 mg
	Fiber	2.8mg
Minerals	Total fats	0.17 mg
	Calcium, Ca	16 mg
	Magnesium	23 mg
	Potassium K	325 mg

	Iron Fe	0.8 mg
	Phosphorus P	40 mg
	Sodium Na	78 mg
	Zinc	0.35 mg
	Copper	0.075 mg
	Vitamin C	4.9 mg
	Vitamin A	33IU
	Folate	109µg
Vitamins	Vitamin E	0.04 µg
	Sugars, total	6.76 gm
	Betaine	128.7 mg
	caffeic acid	0.037 mg
	4-hydroxybenzoic acid	0.012 mg
Phyto-nutriments	chlorogenic acid	0.018 mg
	catechin	0.047 mg
	epicatechin	

According to (Georgiev et al., 2010; Nayik, 2020)

1.5. Bioactive substances in Beta Vulgaris

In the last decade, a burgeoning interest regarding the bio-properties of beets, particularly red beets. Their noteworthy contributions to gut well-being, robust antioxidant efficacy, and wealth of bioactive phenolic compounds have propelled them into the forefront of natural food subjects under intense scrutiny. (Georgiev et al., 2010; Luisa Tesoriere et al., 2004). *Beta vulgaris* is renowned for its abundant reservoir of antioxidants and bioactive compounds, encompassing betalains, carotenoids, phenolic compounds, and various other phytochemical constituents (Gamage et al., 2016; Sasa et al., 2012) Their availability enhances human overall health, supports body organs functionality, promotes longevity and provides defense against various disorders and degenerative conditions. (Azeredo, 2009).

A plethora of studies were performed to identify the components of beetroot and unravel its composition; we cite the work of: Clifford et al., 2015; Guldiken et al., 2016; Lidder & Webb, 2013; Ninfali & Angelino, 2013b; Vulić et al., 2012 whom works identified the following component as present in beetroot: caffeic acid, ferulic acid, p-hydroxybenzoic acid, catechin, rutin, , ascorbic acid, vanillic acid in addition to elements such as carotenoids and betalains, polyphenols,

Saponins, nitrate, and flavonoids, however only small amounts of glycine, folate, minerals, vitamins were confirmed as present in beetroots chemical and biological composition.

1.5.1. Polyphenols and phenolic derivatives

Phenolic compounds found in plants are synthesized from amino acid phenylalanine (Crozier et al., 2006; Manach et al., 2004). As secondary metabolites in fruits and vegetables, these compounds are primarily involved in defense mechanisms against pathogens and/or ultraviolet radiation (Aherne & O'Brien, 2002; Y.-R. Lee et al., 2008; Wootton-Beard & Ryan, 2011). By interacting with proteins, these polyphenols can construct soluble or insoluble complexes, potentially affecting their absorption and biological function (Bandyopadhyay et al., 2012). Koubaier *et al.* identified the following phenolic acids (gallic, vanillic, chlorogenic, ferulic, caffeic, and syringic) in beet stems. This presence gives an explication of the potent antioxidant effect that beetroot holds (Koubaier et al., 2014). However, Gallic acid was the most prevalent trace polyphenol, with concentrations ranging from 11 to 30 mg/L, followed by caffeic acid, syringic acid, and ferulic acid (Wruss et al., 2015).

1.5.2. Flavonoids

The general structure of flavonoids consists of a 15-carbon skeleton, which includes two phenyl rings and a heterocyclic ring. These secondary metabolites are crucial in plants and display highly versatile structures and traits (Saxena et al., 2012). The primary flavonoids in red beetroot are rutin, rhamnocitrin, kaempferol, rhamnetin, and astragalin. While the flavonoids, phenolic amides, and ferulic acids present in the peels and possess antioxidant potential (Kujala et al., 2002; Kujala et al., 2000).

Forward, Koubaier *et al.* identified and confirmed the availability of the following flavonoids: myricetin, quercetin, rutin, and kaempferol in beet stems (Koubaier et al., 2014). Płatosz *et al.* observed that among the flavonoids found in beetroot products (fresh, fermented, and juice), epicatechin and apigenin were more bioavailable than vitexin, rutin, luteolin, quercetin, kaempferol, and orientin (Baião et al., 2017; Płatosz et al., 2020).

1.5.3. Saponins

There is a paucity of studies addressing the quantification of saponin content in beetroot. One of the pillar studies regarding saponins is made by (Koczurkiewicz et al., 2015; Mroczek et al., 2012) that concluded the presence of 11 oleanolic acid-based saponins found in beets are numerable as following (oleanolic acid, hederagenin aglycone, beta vulgarosides I, II, III, IV, V, VI, VII, VIII, IX and X). The saponins present in red beetroot primarily consist of oleanolic acid, These compounds are classified as triterpene glycosides, characterized by the aglycone's covalent linkage to one or two sugar chains through glycosidic ester bonds at position C-28 or ether bonds at position C-3. The structural diversity of these compounds is substantial, and they are ubiquitously found throughout the plant kingdom (Mikołajczyk-Bator et al., 2016). Although proven to be present in beetroots, we find that very few studies have taken them into interest; as a consequence, the knowledge of their presence and properties is scarce.

1.5.4. Betalains

Betalains are nontoxic hydrophilic pigments containing nitrogen. These compounds are constituted by betalamic acid in conjunction with a radical denoted as R1 or R2, with these substituents potentially encompassing either hydrogen atoms or more intricate molecular radicals. Beetroots are recognized as the primary source of betalains and are categorized among the top 10 plants exhibiting the highest antioxidant activity (Vinson et al., 1998). Beetroots have approximately more than 80% of pigments from red beetroot consisting of betacyanins (Liu et al., 2008; Oksuz et al., 2015; Reddy et al., 2005). They are subdivided into two groups based on their chemical composition, structure, and absorbance, to cite:

- (1) *Betacyanins*: red-violet pigment results from the condensation of betalamic acid and cyclo-3,4-dihydroxyphenylalanine (cyclo-DOPA), exhibiting maximum absorption at 538 nm.

Betacyanins are classified into four groups: betanin-type, amaranth-type, gomfrenine-type, and type-2-decarboxylase-nine-nine-nine nine (Ravichandran et al. 2013; Esatbeyoglu et al. 2014).

- (2) *Betaxanthins*: (yellow-orange), Arising from the condensation of betalamic acid ammonium with amines and distinct amino acids, with the maximum absorption at 480 nm.

Betalains are stable over a broad range of pH levels, making them suitable as natural colorants in food products such as yogurt, ice cream, candies, and jellies, available in powder or liquid forms.

Additionally, beet products have been researched for their beneficial impact on hypertension and athletic performance (Muggeridge et al., 2014; Siervo et al., 2013). However, in some extreme cases, betalains can become sensitive molecules if affected by different conditions and have their stability altered. Here are some examples:

- (1) *The pH*: In case of pH less than 4, the absorbance of betalain is greatly diminished in contrast to its absorbance between pH 4-7 (Achparaki et al., 2012).
- (2) *Temperature*: when heating the betalain solution, the red color of betanin starts to diminish, and finally it turns brown. The color loss was followed by the betanin assay by (Achparaki et al., 2012).
- (3) *Light*: Under conditions of 15°C and pH 7, the presence of light heightened the degradation rate by 15.6%, while the presence of air (as opposed to nitrogen) increased it by 14.6% (Harmer, 1980; Tzin & Galili, 2010).

1.5.5. Carotenoids

Beet samples have been recognized for their substantial yield of bioactive compounds, with a predominant presence of carotenoids, principally β -carotene and lutein (Lechner & Stoner, 2019a; Lidder & Webb, 2013). Carotenoids, alternatively referred to as tetraterpenoids, constitute a class of organic pigments. Notably, red beetroot contains β -carotene and lutein among its carotenoid constituents (Lechner & Stoner, 2019b). Nevertheless, following a thorough examination and comparison of existing literature, it becomes evident that comprehensive research on carotenoids extracted from beetroot has been noticeably lacking.

1.5.6. Other Phytochemicals

Beetroot juice phytochemicals reduce and prevents inflammatory illnesses through various signaling pathways (Clifford et al., 2015). The antioxidant compounds in beetroot are believed to function as free radical scavengers, preventing diseases by strengthening the resistance of low-density lipoproteins, DNA, and proteins against oxidative damage (Chang et al., 2008; Kamat et al., 2000; L. Tesoriere et al., 2003; Uttara et al., 2009).

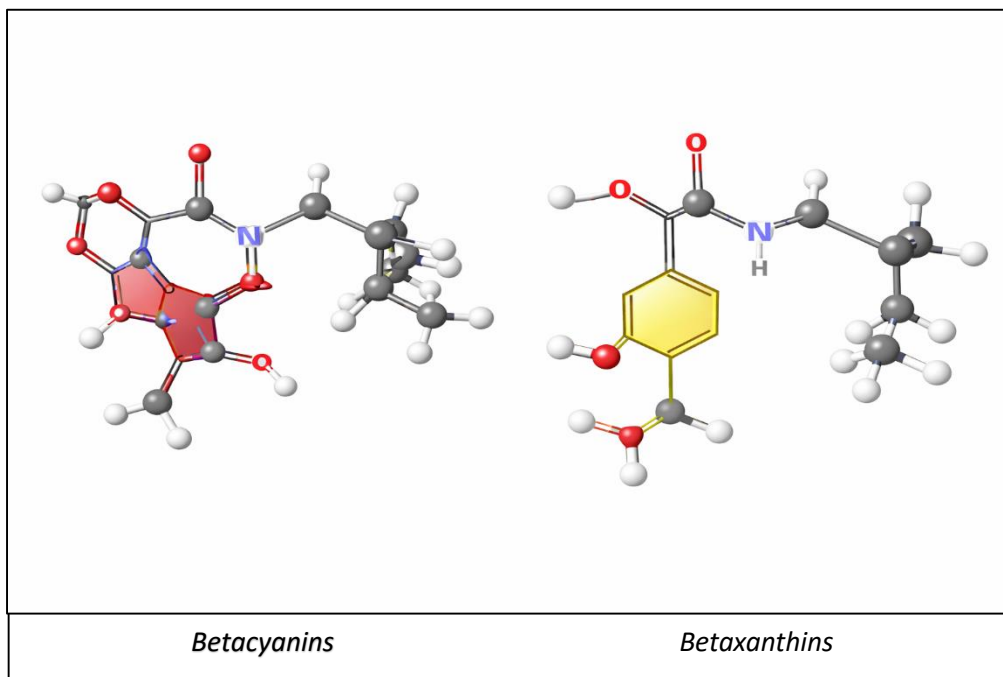


Figure 6:Chemical composition of Betacyanins and Betaxanthin

Source: works of (Esatbeyoglu et al., 2014; Ravichandran et al., 2013)

1.6. Health Benefits of Bioactive Components of Beta Vulgaris

Beetroot exhibits anti-inflammatory potency that help decrease inflammation, a condition closely associated with numerous diseases, including arthritis and heart disease. Regular consumption of beetroot also supports liver detoxification, while its high vitamin C and folate content enhances immune system function. The cardiovascular benefits of beetroot, notably its ability to lower blood pressure, are extensively studied, positioning it as an essential food for preserving the heart health (Stoica et al., 2025)

The therapeutic potential of beetroot as a dietary intervention in various pathologies, particularly those associated with oxidative stress, inflammatory response, and other diet-related diseases is well noted. This therapeutic application is primarily attributed to betalains, which have shown significant antioxidant, anti-inflammatory, and chemo preventive activities in both in vitro and in vivo studies (Clifford et al., 2015; Lechner & Stoner, 2019a; Rahimi et al., 2019). Beetroot also contains a wide range of bioactive molecules that may promote health and help prevent

diseases such as high blood pressure, cardiovascular complications, and chronic inflammation, further enhancing its role as a functional food for disease prevention.

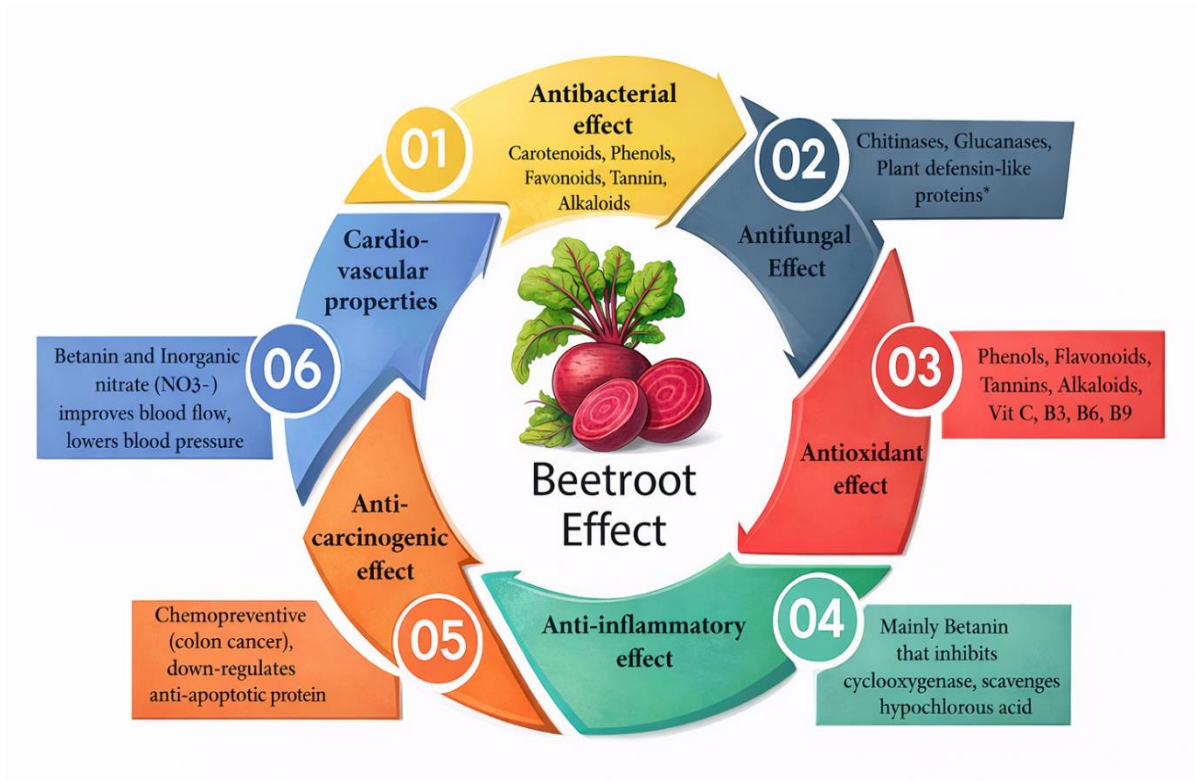


Figure 7. Different effects provided by beetroot consumption for the human health

Source (Clifford et al., 2015; Lechner & Stoner, 2019a; Rahimi et al., 2019)

1.7. Antioxidant properties

Bioactive elements may exhibit diverse physiological goals and pathways of action. A significant subset of these compounds demonstrates antioxidant properties through their capacity to engage in oxidation/reduction processes with specific molecules (Liu, 2004). Protection against oxidative damage is a known mechanism involved in cellular activity. Oxidative damage triggers the release of reactive oxygen species (ROS or free radicals) during cell metabolism. At low levels, ROS play a protective role in the cellular system, contributing to muscle contraction, cell proliferation, gene expression, and apoptosis. Antioxidants are also needed when the formation of free radicals, such as reactive nitrogen species (RNS), occurs as a by-product of cellular metabolism and is generated inside the body, as the first line of defence (del Río et al., 2006; Fang

et al., 2002; Lobo et al., 2010). Halliwell & Gutteridge (2015 defined a molecule as possessing antioxidant properties if it exhibits the capability to retard, avert, or ameliorate oxidative damage to specific target molecules (Nayik, 2020; Pedreño & Escribano, 2001; Vinson et al., 1998; Vulić et al., 2012).

1.7.1. Antibacterial effect

Beetroot extract exhibits antioxidant properties and demonstrates antimicrobial activity against both Gram-positive and Gram-negative bacteria. Notably, Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus cereus* exhibit greater susceptibility to the extract than Gram-negative strains like *Escherichia coli* and *Pseudomonas Typhimurium* (El-Beltagi et al., 2018). In the study of Maqbool et al. 2020, their results showed that the direct crude extract of beetroot peel has antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, and *Vibrio cholera*. The highest inhibition was against *E. coli*, and the minimum was against *Salmonella typhi*, similar to the finding of (Narender et al., 2018).

1.7.2. Antifungal and anti-mycotoxigenic effects of beetroots

Researchers have proposed different explanations for the antifungal abilities of some plants, especially beetroots. One of them is the presence of chitinase enzymes that can catalyse the hydrolysis of chitin, a homo-polymer of L-1,4-N-acetyl-D-glucosamine, which is a structural component of the cell wall of true fungi (Bartnicki-Garcia, 1968). It was the suggestion from Hirano & Nagao, 1989, and Sekiguchi et al., 1994, explaining that low molecular weight chitosan in an agar system inhibited a range of phytopathogenic fungi more effectively than high molecular weight chitosan inhibited the organisms, thus supporting the claim that the presence of Chitinases confers partly that antifungal effect.

On those same bases, the investigation of the interaction between beetroot (*Beta vulgaris L.*) and the pathogen *Cercospora beticola* (Sacc.) that causes leaf spot disease in beet was highlighted. Previously, some Chitinases, Glucanases, and Plant defensin-like proteins have been isolated and their biological role examined. Chitinases that are secreted extracellularly and deposited in the apoplast inhibit the growth of the conifer pathogen (Berglund et al., 1995; Mikkelsen et al., 1992; Neuhaus et al., 1991; NIELSEN et al., 1994; Susi et al., 1995).

1.7.3. Anti-inflammatory effect

Inflammation is a protective response and a multifaceted physiological process triggered by harmful stimuli and conditions (such as tissue damage), which are linked to pathogen-associated molecular patterns and antigens. Betanin, the predominant betalain in beetroot, has been noted to exhibit anti-inflammatory traits by inhibiting cyclooxygenase, scavenging hypochlorous acid, and neutralizing oxidants produced by neutrophils during the inflammatory response (Allegra et al., 2005; Reddy et al., 2005). The effects of natural food colors namely betanin, anthocyanin, lycopene, bixin, β -carotene, and chlorophyll on lipid peroxidation and cyclooxygenase enzyme inhibition in human tumor cells were assessed in a particular study. It showcased that, betanin, cyanidin-3-O-glucoside, lycopene, and β -carotene inhibited lipid peroxidation. However, all pigments tested showed a dose-dependent aspect (Reddy et al., 2005). Kujawska et al. (Kujawska et al., 2009) demonstrated through their study on the anti-inflammatory effect of beetroot and some of its derivatives that a positive effect on stress and inflammation has been reduced.

1.7.4. Cardiovascular disease prevention

Epidemiological studies have shown that vegetables are useful protective foods against coronary heart disease and ischemia (Joshi et al., 2001). Since the administration of NO₃ supplementation as beetroot juice enhances cardio-protective and cardio-enhancing properties by the works of (Hendgen-Cotta et al., 2012; Larsen et al., 2007; Loscalzo, 1992). A study involving 46 healthy participants administered either 100 mL of beetroot juice or a placebo (equivalent to 400 mg and 2 mg of NO₃, respectively). The results were, an increase in salivary nitrate levels in both groups, with the beetroot juice group exhibiting a more significant rise. It was noted that the highest baseline value was reached on day 8 for the beetroot juice group and on day 15 for the placebo group (Hohensinn et al., 2016).

1.7.5. Beetroot Effect on Gut Microbiome

While data on the effect of *Beta vulgaris* on the gut microbiome and salivary microflora is limited, its potential is linked with metabolic dysfunction should not be overlooked. In this context, it was initially proposed that dietary NO₃ supplementation could influence the salivary microbiome, this hypothesis was then investigated and reliable evidence indicates that non-sucrose polysaccharides, particularly pectin and pectic oligosaccharides outsourced from beet, have a

beneficial consequences on the gut microbiota's composition and function. (de Oliveira et al., 2021; Mirmiran et al., 2020). Commensal bacteria residing in the oral cavity that express the enzyme nitrate reductase can reduce Dietary nitrate (NO_3^-) into nitrite (NO_2^-) (Lundberg et al., 2008, 2009). When bioconversion is assumed to occur in the mouth, the consumption of nitrate-rich *Beta vulgaris* increases both the rate of NO bioconversion and pH levels. This process may be vital for host defense, lowering the prevalence of metabolic dysfunction and caries in the oral cavity, due to the acid-neutralizing effects of human saliva and its impact on the microbiome composition (Duncan et al., 1995; Hohensinn et al., 2016; Jones-Carson et al., 1995).

Table 2. Studies on the beneficial health effects of beetroot (*Beta vulgaris*) based on literature data.

Bioactive role	compounds	Proposed mechanism	References
Antioxidant	Rutin	<ul style="list-style-type: none"> Keeping the reactive oxygen species (ROS) at a low level 	(Faggian et al., 2016; Georgiev et al., 2010; Kujawska et al., 2009; Vulić et al., 2012)
	Kaempferol		
	Rhamnetin	<ul style="list-style-type: none"> Stop the stimulus causing imbalanced redox homeostasis 	
	Rhamnocitrin		
	Astragalin		
Betalain			
Anti-carcinogenic	Betaxanthins	<ul style="list-style-type: none"> Betalains reduce the expression of pro-inflammatory markers 	(Farabegoli et al., 2017)
	Betacyanins	<ul style="list-style-type: none"> Causes down-regulation of the anti-apoptotic protein 	
Anti-inflammatory	Betanin	<ul style="list-style-type: none"> Counteracts xenobiotic-induced oxidative stress. Restores the activity of major antioxidant enzymes in the liver Reduces oxidative injury to plasma proteins. 	(Kujawska et al., 2009)

		<ul style="list-style-type: none"> Decreases DNA damage in leukocytes. 	
Antibacterial	carotenoids, phenols, flavonoids, tannins, alkaloids	<ul style="list-style-type: none"> Higher effectiveness against Gram-positive than Gram-negative. The inhibitory molecule is most likely hydro-soluble 	(El-Beltagi et al., 2018; Maqbool et al., 2020; Narender et al., 2018)
Antifungal	Chitinases enzymes	<ul style="list-style-type: none"> Chitinase catalyzes the hydrolysis of chitin, which is a structural component of the cell wall of true fungi. Inhibiting the growth of the conifer pathogen. 	(Berglund et al., 1995; Mikkelsen et al., 1992; N. H. Youssef et al., 2021)
Prevent cardiovascular diseases	High Inorganic Nitrate presence	<ul style="list-style-type: none"> Inorganic nitrate (NO_3^-) acts as a substrate for nitric oxide (NO) production. Nitric oxide (NO) induces vasodilation and reduces blood pressure. Supports cardiovascular function. Contributes to the prevention of coronary heart disease and ischemia. 	(Hendgen-Cotta et al., 2012; Larsen et al., 2007; Loscalzo, 1992; Lundberg et al., 2009)

<p>Gut microbiome modulation of pectin and pectic oligosaccharides</p>	<ul style="list-style-type: none"> • Positive modulation of gut microbiota composition and function. • Bioconversion of bioactive nitrogen oxides through the NO₃⁻-NO₂/NO pathway. • Lower prevalence of metabolic dysfunction. • Reduced the occurrence of dental caries in the oral cavity. • Acidification-preventing properties of human saliva. 	<p>(Mirmiran et al., 2020) (de Oliveira et al., 2021)</p>
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1.8. Biotechnological usage of *Beta Vulgaris*

Beetroot is traditionally ingested as boiled, oven-dried, pickled, pureed, supplemental juice, powder or jam-processed across multiple cultures. It's highly regarded as a nutritious stem since it confers a multitude of nutrients that our daily intake lacks due to clearances and non-equilibrated diets.

1.8.1. Functional food involvement

Beetroot is regarded as a functional food due to its high levels of vitamins (K, C, A, B6, thiamin, and E), minerals (calcium, iron, magnesium, potassium, selenium, and zinc), along with phenolic compounds, carotenoids, ascorbic acids, and betalains, which support human health (Chhikara et al., 2019). In contrast to other fruits, the main sugar in beetroot is sucrose with only small amounts of glucose and fructose (Bavec et al., 2010).

The food industry has commercially utilized these red beet pigments to enhance the color of a variety of products, including dairy items (yogurt, ice cream, milk, kefir, sandwiches), sauces (tomato pastes), soups, candies (jams, jellies, desserts, cookies), breakfast cereals, processed meats, and cattle products (smoked, cooked, fermented, or semi-dry sausages)(Azeredo, 2009; M.

Bandyopadhyay et al., 2007; Cai et al., 2005; PASCH et al., 1975; Singh & Hathan, 2017). Several new formulations of beetroot have been proposed through several different processes, among which:

- (1) *Red round thin beetroot chips*: a trending new functional form of beetroot supplements, they have been proven to contain the highest energy content (Kcal), carbohydrate, and total sugar, the highest value of Total Antioxidant Potential (TAP), however, they scored the lowest value of Total Phenolic Content (TPC), flavonoids and Saponins level (Vasconcellos et al., 2016).
- (2) *Pseudo-plastic beetroot gels*: The pseudo-plastic gel is a means of administering NO₃⁻ for athletes, it contains the highest protein and lowest lipid content, ranking beetroot gel not as the most commonly used but the most effective formulation compared to other beetroot by-products. The beetroot gel was formulated with a mixture of beetroot juice, beetroot powder, and carboxy-methylcellulose at 90:17:3 ratios (Baião et al., 2017).

1.8.2. Beetroot juice

Beetroot juice is packed with antioxidants that help reduce inflammation and blood pressure, and is now recommended for athletes to enhance their performance. With its high nutritional value, beetroot juice is considered a vegetable that promotes health. However, most studies have concentrated on the benefits of beetroot juice rather than its raw or cooked versions. This is because beetroot's nitrate content is converted into nitric oxide in the digestive system, which dilates blood vessels, reduces blood pressure, and enhances blood flow.(Nayik, 2020).

Wootton-Beard and Ryan. (Wootton-Beard & Ryan, 2011) identified the increase in simulated digestion as a function of antioxidants by the consumption of beetroot juice, and also confirmed that the juice components are structurally altered phytochemicals that show similar functions. Stated that an administration of 8 ml/kg body weight/day of beetroot juice lowered protein oxidation, DNA damage, and lipid peroxidation in rats.

To the best of our knowledge. Natalia et al. (2020) For the first time, the effects of fermentation on the phenolic acid and flavonoid content of red beets were studied, alongside the impact of consistent, long-term consumption of this vegetable on the phenolic profiles in plasma and urine

of volunteers. beetroot revealed to be a notable source of phenolic acids and flavonoids. The findings highlight that fermentation increased the content of free phenolic acids while reducing the levels of conjugated phenolic acids (Panghal et al., 2017).

Table 3. Relevant works reporting beetroot product involvement as a supplementary food or as a health-promoting food

Product Characteristic	Finding	References
Beetroot juice	Lowered blood pressure by 5.2 ml /24 h	(Bondonno et al., 2015)
	Contains betalains, betacyanins (betanin and Isobetanin), and protects the individual against damage to DNA, protein, and lipid.	(Kujawska et al., 2009; Winkler et al., 2005)
	Significantly increased workload capacity	(Pinna et al., 2014)
Lacto-fermented Beetroot Juice	Contains the <i>lactobacillus</i> genus, conferring limitation of oxidative processes.	(Klewicka et al., 2012)
	Reduced the level of ammonia by 17% in the tested rats. Observed positive modulation of the gut microflora and its metabolic activity.	
Beetroot juice	Enhancement of Cardio-respiratory endurance. Nitrate is reduced to nitrite and then to nitric oxide (NO). Acting on muscles to confer enhanced performance	(Domínguez et al., 2017)
Beetroot juice and black tea	led to a decrease in Peripheral vascular resistance at the resistance vessels only. Postprandial forearm and leg resistance	(Fuchs et al., 2016)

Beetroot salad	Excellent food in pregnancy: promotes the growth of the fetus through the presence of multivitamins.	(Chawla et al., 2016)
Beetroot chips	Enriched in flavonoid content high in Total Phenolic (292mg/100 g)	(Kaimainen et al., 2015; Ninfali & Angelino, 2013a; Vasconcellos et al., 2016)
Beetroot chips	High value in total antioxidant potential (TAP) and a low total phenolic content (TPC)	(Baião et al., 2017)
Beetroot gel	Aimed to administer NO ₃ ⁻ to athletes during sports competitions. Formulated a mixture of beetroot juice, beetroot powder, and Carboxymethylcellulose at a 90:17:3 ratio. Significantly higher levels of NO ₃ ⁻ and bioactive compounds compared to other formulations.	(Vasconcellos et al., 2016) (Silva et al., 2016)
Beetroot yogurt sweetened with stevia	<ul style="list-style-type: none"> Stimulated the development of probiotic bacteria. <p>Growth proportion index (GPI) of <i>L. casei</i> in beetroot yogurt remained stable after fermentation.</p> <p>Beetroot and stevia yogurt exhibited high techno-functional and nutraceutical properties.</p> <p>Associated with higher fiber consumption and lower energy intake</p>	(Ozcan et al., 2021)

Beetroot (*Beta vulgaris*) has garnered attention as a potential alternative to traditional synthetic media for cultivating lactic acid bacteria (LAB). Its rich nutritional profile, including sugars, amino acids, and micronutrients, makes it a promising candidate for supporting microbial growth. Studies have demonstrated that beetroot juice can serve as a substrate for various LAB strains, promoting their proliferation and lactic acid production. For instance, research by Kyung et al. (2005) highlighted that *Lactobacillus plantarum* and *Lactobacillus acidophilus* could effectively ferment beet juice, produce significant amounts of lactic acid, and reduce the pH to below 4.5 within 48 hours of fermentation.

The incorporation of beetroot pulp into fermentation processes has also been explored to enhance the growth of LAB. A study by Silva et al. (2020) investigated the use of pre-fermented sugar beet pulp as a growth medium for *Pleurotus ostreatus* mycelium, noting that pre-fermentation with LAB improved the pulp's suitability for microbial growth. This approach suggests that beetroot pulp, when pre-fermented, could provide a conducive environment for LAB cultivation, potentially offering a cost-effective and sustainable alternative to commercial media (Silva et al., 2020).

In addition to its nutritional benefits, beetroot-based media offer environmental advantages. Utilizing beetroot and its by-products for LAB cultivation aligns with sustainable practices by reducing reliance on synthetic media and minimizing waste

Chapter 2

Biotechnological and health

benefits of Carob

(Ceratonia siliqua L)

Carrober: Chemical Profile and Biotechnological Potential

1.1. Introduction about *Ceratonia siliqua L*

The carob tree (*Ceratonia siliqua L.*) is a species of evergreen plant belonging to the family *Fabaceae*, subfamily *Caesalpinioideae*. Carob trees can withstand long periods of drought, allowing them to grow in very harsh environments and making them a highly productive species widely grown in several countries worldwide (Hadi et al., 2017). The fruit of the carob tree is highly prized, and its pods are a luscious dark brown and may be long, straight, or curved. These pods are mainly made of pulp (90%), and 8% to 10% derived from seeds, which contain bark, endosperm, and germ (Ben Ayache et al., 2021).

Although antioxidant and phenolic contents of carob pods have been widely reported in the literature, a number of properties related to their biological activities still need further investigation. The purpose of this chapter is to address a complete review, with special interest in the chemical composition, bioactive compounds, therapeutic effects, and biotechnological and food model applications of Carob (*Ceratonia siliqua L*). By reviewing and comparing different resources, we shall also consider what it means as well as its purposes, past uses, and relevance to this chapter (Cakilcioglu et al., 2011; Custódio et al., 2013; Hayes et al., 2018; Karioti et al., 2010; Mohammad & Kowluru, 2012)

1.2. Botanical classification

- Kingdom: *Plantae* (Plants)
- Division: *Angiosperms* (Flowering plants)
- Class: *Eudicots*
- Order: *Fabales*
- Family: *Fabaceae* (Legume family)
- Subfamily: *Caesalpinioideae*
- Tribe: *Cercidoideae*
- Genus: *Ceratonia*

- Species : *Ceratonia siliqua* (Carob tree)



Figure 8. Carob pod sample

1.2.1. Geographical distribution according to (POWO, 2025)(Batlle et al., 1997)

Ceratonia siliqua, known also as the carob tree, is native to the Mediterranean region, where it prospers in rocky, dry soils and warm climates. Gradually, it spread to other regions with conditions, including North Africa, the Middle East, and even Australia. (For a clearer perspective of its spreading, refer to Figure 9).

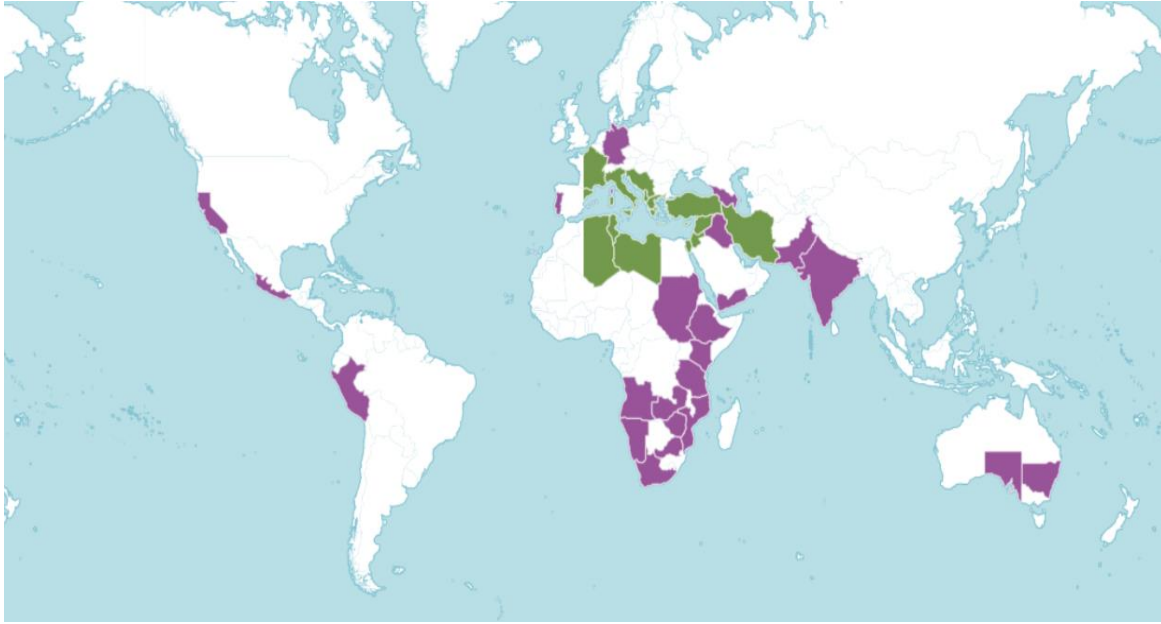


Figure 9. Distribution of *Beta vulgaris* Worldwide Green: Native Violet: introduced to

1.3. Carob Cultivation Regions in Algeria

Carob (*Ceratonia siliqua*) is a drought-resistant tree that thrives in various regions of Algeria, especially in the Tell Atlas and coastal areas. Key regions include:

1. **Tlemcen:** Situated in the northwest.
2. **Ain-Temouchent:** Located to the west of Algiers.
3. **Sidi Bel Abbès:** In the northwestern part of Algeria.
4. **Blida:** Near Algiers, Blida is another region where carob trees are cultivated.
5. **Chelef** – Located to the west of Algiers.



Figure 10. The map showcases the five regions in Algeria where carob trees are cultivated: Tlemcen, Aïn-Temouchent, Sidi Bel Abbès, Blida, and Chelef.

1.4. Chemical composition of Carrober

Carob (*Ceratonia siliqua L.*), a leguminous tree native to the Mediterranean and Middle Eastern regions, has garnered increasing scientific and commercial attention due to its rich nutritional composition and versatile functional applications. The pulp of the carob, which makes up nearly 90% of the fruit, is rich in sucrose, contributing to its natural sweetness. The other 10% of the fruit, which, on a structural level, is comprised of seeds made up of three parts: a seed coat, germ, and endosperm. Due to its sticky texture and high sugar content, it is commonly used as a natural sweetener in its native regions (Mahtout et al., 2018). Traditionally employed as a natural sweetener and cocoa substitute, carob is particularly valued for its high carbohydrate content, low fat levels, and absence of caffeine and theobromine. Its macronutrient profile is dominated by carbohydrates (mean value: 75.92%), accompanied by a modest but nutritionally valuable protein content (6.34%) and low-fat content (1.99%) (GHANEMI & BELARBI, 2021). The presence of crude fiber (7.30%) contributes to digestive health and enhances satiety, while its caloric value, approximately 346.95 kcal per 100 g of dry matter, supports its classification as an energy-dense

plant-based food. In addition to macronutrients, carob pods are rich in bioactive compounds, dietary fiber, and essential micronutrients, reinforcing their significance as a health-promoting food supplement in both traditional diets and contemporary functional food formulations (Bartkiene et al., 2019; Kaur et al., 2013). Moreover, the chemical characterization of carob seeds reveals their content in various biologically active compounds. Carob seeds exhibit a high content of γ -tocopherol (20.2 $\mu\text{g/g}$ dw), contributing significantly to the total tocopherol content (30 $\mu\text{g/g}$ dw). The presence of tocopherols, including α -, β -, γ -, and δ -tocopherol, establishes carob seeds as a source of vitamin E vitamers, underscoring their bioactive potential (Biernacka et al., 2017; GAITA et al., 2020; Ortega et al., 2011)

1.5. Bioactive Components of Carob

1.5.1. Carbohydrate and Fiber Composition

Carob pods are composed of approximately 70–80% carbohydrates by dry weight, predominantly sucrose, glucose, and galactose in a 7:2:1 ratio. These sugars undergo compositional changes during maturation, with immature pods being less sweet and traditionally incorporated in North African dishes, while fully mature pods are widely used in natural sweeteners and confectionery. Beyond sugars, carob is an exceptional source of dietary fiber, particularly soluble fiber extracted from the endosperm in the form of locust bean gum. This soluble fraction exhibits excellent gelling and binding properties, enhancing its application in both food and pharmaceutical formulations. Approximately 47% of the total dietary fiber in carob is soluble, and its composition may vary with origin and extraction method (Bartkiene et al., 2019; Dakia et al., 2008)

1.5.2. Protein and Amino Acid

While not a complete protein source, carob exhibits a protein content of approximately 8%, exceeding that of many cereals. Its amino acid profile is characterized by high levels of glutamic acid, arginine, and leucine. Essential amino acids constitute up to 45% of its total amino acid content, with phenylalanine and tryptophan among the most abundant, both of which may contribute to its naturally sweet flavor. However, lysine—a key amino acid in legume nutrition—is present in relatively low quantities. Advanced amino acid profiling has detected 14 amino acids in carob, with histidine and phenylalanine identified as first-limiting amino acids. Due to the high

phenylalanine content, consumption in children with metabolic sensitivities should be approached cautiously (Bengoechea et al., 2008).

1.5.3. Lipid Composition

Though carob pods are low in fat, carob seeds contain a modest lipid fraction (~2.1%), enriched with health-promoting compounds such as sterols, tocopherols, and phospholipids. β -Sitosterol dominates the sterol profile, comprising approximately 74.2% of the total, followed by stigmasterol. These sterols have well-documented cholesterol-lowering, antioxidant, and anti-inflammatory properties. Tocopherols, particularly γ -tocopherol and α -tocopherol, represent 20.2 $\mu\text{g/g}$ and 10 $\mu\text{g/g}$, respectively, contributing significantly to carob's oxidative stability (Matthaus & Özcan, 2011; Musa Özcan et al., 2007).

1.5.4. Fatty Acid Profile and Health Implications

Carob seed oil exhibits a favorable fatty acid composition, Over 78% of the total lipid content consists of unsaturated fatty acids, with oleic acid (45.0%) and linoleic acid (32.4%) being the major unsaturated fatty acids. The relatively low saturated fat content (21.9%) and high omega-6 concentration suggest potential cardiovascular benefits. Notably, the oleic acid content in carob oil exceeds levels reported in other legumes, indicating genetic or environmental influences (Fidan et al., 2020).

1.5.5. Polysaccharides and Functional Carbohydrates

Carob seed polysaccharides are primarily composed of mannose and galactose, which together form galactomannans—valuable hydrocolloids used in food and industrial applications. The mannose-to-glucose ratio of 3.5 confirms a high galactomannan content, making carob seed extracts highly desirable for textural and stabilizing roles in food systems. Quantitative sugar analysis reveals the presence of sucrose (8.1%), glucose (2.2%), mannose (54.0%), and galactose (15.5%), among others. These compounds enhance the functional and prebiotic potential of carob in the human diet (Correia et al., 2018).

1.5.6. Vitamins, Minerals, and Micronutrients

Carob pods are modest sources of vitamins such as B1, B2, B6, vitamin A, and vitamin E. While their concentrations do not generally meet daily recommended intake levels, the presence of essential minerals, including potassium, calcium, magnesium, and iron, adds to carob's nutritional profile. The concentration of these micronutrients is influenced by genetic variation, growing conditions, and post-harvest handling, leading to variability in nutritional outcomes. Additionally, the polyphenolic content of carob enhances its functional value by contributing antioxidant capacity and digestive benefits (Ait Ouahioune et al., 2022; Correia et al., 2018).

1.5.7. Mineral content

Table 4. Mean values of major chemical components in Carob from different geographical locations (Correia et al., 2018)

Chemical Component	Content (mean \pm SD)
Moisture (%)	9.2 \pm 0.3
Protein (%)	3.8 \pm 0.2
Crude Fat (%)	0.6 \pm 0.1
Ash (%)	2.6 \pm 0.2
Crude Fiber (%)	6.2 \pm 0.3
Carbohydrates (%)	77.6 \pm 1.2
Caloric Value (Kcal/100g)	319 \pm 4

The mineral content of carob seeds was evaluated for two macronutrients (Ca and Mg) and four micronutrients (Fe, Cu, Mn, B, and Zn). The findings suggest that carob seeds can be considered a source of calcium and magnesium. The amounts of other minerals were significantly lower, with manganese and zinc concentrations being quite low, and iron (Fe) and copper (Cu) present in moderate amounts., Fidan et al., (2020) findings align with those reported from Bouzdoudi et al., (2017) all are expressed in Table 4.

1.6. Health Benefits of Bioactive Substances in Carob

1.6.1. Exploration of the Medicinal Properties of Carob

Ceratonia siliqua exhibits health-enhancing potential and has been employed in traditional medicine for the treatment of digestive diseases, and its dietary fiber has been associated with enhanced GI function and health (Azimova & Glushenkova, 2012). Carob's antioxidant and anti-inflammatory effects have been highlighted by recent research, which are mainly due to its polyphenols, tocopherols, and other bioactive substances. These products have proved to be powerful protectors against oxidative stress associated with the development of chronic diseases, such as cardiovascular diseases, metabolic syndromes, and cancer. Growing scientific evidence can be used to support the incorporation of carob in modern therapeutics, fuelling usages as a functional food and traditional cure (Babiker et al., 2020)

1.6.2. Traditional Uses in Folk Medicine

Historically, carob has been used in traditional medicine as a remedy against diarrhea and for its antiseptic and anti-inflammatory activities, also used medicinally as a remedy to help support respiratory function and heart health. It was also popular for use as a natural way to calm coughs, mainly in the Mediterranean area. The cultural and historical importance of carob within herbal medicine is a good argument for further investigation of its bioactive effects and possible modern development.

1.6.3. Connection to Various Health Conditions

The potential therapeutic applications of carob extend beyond traditional uses, with modern research establishing its connection to a range of health conditions.

Key Health Conditions Linked to Carob Consumption:

- **Diabetes Management:** Carob fiber and polyphenols slow glucose absorption, potentially lowering postprandial blood sugar levels.
- **Weight Management & Obesity Prevention:** High fiber content contributes to satiety and digestive regulation, promoting healthy weight maintenance.

- **Cardiovascular Protection:** Plant sterols and polyphenols support heart health by lowering LDL cholesterol and improving blood circulation.
- **Neuroprotective Effects:** Antioxidants in carob may reduce oxidative stress in brain cells, potentially aiding in neurodegenerative disease prevention.

Research by Fidan et al. (2020) identified the presence of four tocopherol vitamers (α -, β -, γ -, and δ -tocopherol) in commercial carob seeds from Turkey, further validating its nutritional and therapeutic significance.

1.6.4. Antioxidant Properties

Antioxidant activity plays a fundamental role in the body's defense mechanisms against oxidative stress, which results from an excess of reactive oxygen species (ROS). These oxidant agents, originating from environmental pollutants, UV radiation, and metabolic processes, contribute to cellular damage and the progression of chronic diseases. Given the link between diet and antioxidant intake, the search for natural, plant-derived antioxidants has gained significant attention in recent years (Correia et al., 2018). Carob has been identified as a rich source of polyphenols, a class of bioactive compounds known for their potent antioxidant activity. Although the full extent of carob's antioxidant potential has not yet been completely established, studies indicate that polyphenols in carob play a crucial role in reducing oxidative damage in the body. These compounds function by neutralizing free radicals, thereby preventing lipid peroxidation and DNA damage—two key processes implicated in disease onset and aging. A particularly noteworthy study examined the effect of carob polyphenols on oxidized low-density lipoproteins (LDL), a key factor in atherosclerosis development. The results demonstrated that carob-derived polyphenols significantly inhibited the formation of conjugated dienes early and reduced the uptake of carbazole, a compound involved in oxidative stress pathways (Tesoriere et al., 2003).

1.6.5. Protecting Against Chronic Diseases

A high intake of dietary antioxidants, such as those found in carob, may play a crucial role in preventing chronic diseases. Substantial scientific evidence suggests that diets rich in fruits and vegetables are associated with a lower risk of chronic conditions, including cardiovascular disease (CVD), diabetes, and neurodegenerative disorders. Carob has been identified as a particularly rich

source of antioxidant compounds, with studies reporting notable polyphenol concentrations in various carob-based products (Aziz & Hicham, 2014): Carob syrup: 957 mg/100 g, Carob powder: 326 mg/100 g, Carob seeds: 272 mg/100 g.

Carob products has been assessed using the diphenylpicrylhydrazyl (DPPH) radical scavenging method, showing that carob syrups and pod flours exhibit greater antioxidant capacity than many commercially available natural sweeteners. This suggests that carob-based products may serve as nutritional enhancers in food formulations, offering superior health benefits compared to conventional sweeteners (Pazir & Alper, 2018). One of the most significant antioxidant compounds in carob is proanthocyanidins, a type of condensed tannin with wide-ranging health-protective effects. These compounds have been shown to inhibit key enzymes involved in degenerative and inflammatory conditions. For instance:

- Inhibition of prolyl and hydroxyprolyl endopeptidase may slow collagen degradation, potentially benefiting individuals with degenerative joint diseases.
- Regulation of vascular permeability could reduce vascular fragility, which is implicated in certain inflammatory conditions.

Furthermore, carob proanthocyanidins have been suggested as potential agents in asthma prevention, as reported in Volume 33 of *Pharmacognosy Communications*.

1.6.6. Enhancing Immune Function

The immunostimulatory properties of carob remain a relatively underexplored area, but emerging evidence suggests that its bioactive compounds may have a positive impact on immune health. While the antioxidant and anti-inflammatory effects of carob indirectly enhance immune function by reducing disease burden and oxidative stress, some studies have directly linked carob consumption to improved immune response.

Early animal studies have provided promising insights:

- Rats fed a diet rich in carob flour exhibited higher T-cell counts and an improved mitogen response, suggesting enhanced adaptive immunity.

- Another study found that rats consuming locust bean gum (a dietary fiber derived from carob) displayed stronger antibody responses to a hepatitis vaccine, indicating an improved immune defense mechanism.

1.7. Biotechnological usage of Carob

1.7.1. Applications in the Food Industry

Applications The fermented Carrobier product has been applied in the food industry, especially in the development of functional foods with added nutritional values. Fermented Carrobier, because of its probiotic characteristics, is also used more and more in the fermentative part of the production of probiotic beverages (like kefir-type drinks) and fermented foods as snacks (Tsatsaragkou et al., 2014). Not just good for essential nutrition, BAs may also benefit gut health with live LAB-based cultures. The increasing request of consumers for plant-based and functional foods caused Carrobier to be exploited in the development of plant-based products, such as dairy alternatives, energy bars, or health supplements (Ait Ouahioune et al., 2022). These products are appreciated due to their high nutritional value (rich in bioactive compounds, health-promoting), thus fermented Carrobier might be a prospective ingredient for the plant-based food industry.

Functional nutraceuticals Fermented Carrobier is also rich in the key ingredients for a diet under consideration as a functional food, such as polyphenols and dietary fiber, in addition to being a probiotic (Liu et al., 2021).

1.7.2. Cosmetic and Pharmaceutical Applications

The beauty industry also already started to analyze fermented Carrobier, mainly for its antioxidant and anti-aging activities. Due to LAB fermentation of the carobier, the final carob-paste can be used as an active ingredient for rich-antioxidant creams, lotions, and serums. These health-promoting compounds prevent oxidant damage and environmental stresses to skin and therefore help retard early aging (Gänzle et al., 2016). In addition, the anti-inflammatory properties of LAB-fermented carobier could be important in the beer as a strategy to treat skin diseases like eczema and psoriasis, where inflammation is a cornerstone of the disease (Fukai et al., 2020). The fermentability of carobier can be used not only in cosmetics but also in medicine. Further, its bioactive metabolites following carobier fermentation, namely flavonoids and phenolic acids--

have also been studied as potential natural products in the development of new drugs by showing anti-inflammatory, antioxidant, and antidiabetic properties (Patel et al., 2017).

1.7.3. Carob's Role in Sustainability in Biotechnology

Carobier is a game-changing company in advancing sustainability in the biotech space, primarily due to its low environmental footprint and local agricultural opportunities. Being a drought-resistant plant that grows in arid areas makes carobier need little water and fertilizer sources, and it becomes an environmentally friendly crop while producing a functional food product and bioactives (Gänzle, 2019). From the biotechnological point of view, carobier is also in line with those principles envisaged by green chemistry and sustainable resource exploitation. Its application in the food and pharma industries reduces the dependency on synthetic chemicals, contributes to using natural, renewable resources (Banwo et al., 2020).

One of the key advantages of carob-based innovations is their suitability for individuals with dietary restrictions, such as those with nut allergies or celiac disease. By developing new carob-based food products, industries can provide safe and nutritious alternatives while simultaneously supporting carob producers and enhancing market demand (Goulas et al., 2016). In regions such as Egypt, carob pods are commonly incorporated into cakes, cookies, beverages, and snacks. In countries like Turkey, Malta, Portugal, Spain, and Sicily, carob is used to produce jams and liquors, while in Libya, a traditional carob-based syrup known as "rub" is used to prepare asida, a traditional dessert (López-Sánchez et al., 2018; Özcan et al., 2009). Carob pods are increasingly recognized as a nutrient-rich component for human diets and are commonly used as livestock feed. In the culinary sector, carob is widely used in the production of sweets, biscuits, glazes, and chocolate substitutes (Pazir & Alper, 2018).

1.7.4. Culinary Usages of Carob

Carob powder and carob chips have long found their way into a baker's kitchen as a chocolate substitute. Because it is naturally sweeter, for recipes that call for carob chips. Moreover, carob bakes more quickly than cocoa; hence, one has to decrease the baking temperature by 25°F (ca. 10°C) if one does not want the product to be burned (Youssef et al., 2013). Cookies and cakes are enhanced by Carob as it provides a mild, caramelly flavor. (Loullis & Pinakoulaki, 2018).

Carob makes a great cocoa substitute in puddings and custards using pre-mixed or homemade recipes. In which the carob pudding is prepared by mixing sugar, cornstarch, milk, and carob powder in a mix that is cooked until thick (Pazir & Alper, 2018). Carob pods taste sweet and can be a substitute for cacao powder in most recipes, making a safer choice for people who need to avoid those substances as part of their diet, such as children, the elderly, or those with anxiety disorder (GHANEMI & Meriem BELARBI, 2021)

Carob drink is a seasonal beverage in North Africa and the Mediterranean, where it is consumed during Ramadan due to its hydrating and energizing effects. Of course, it's caffeine-free and also full of antioxidants along with crucial minerals.

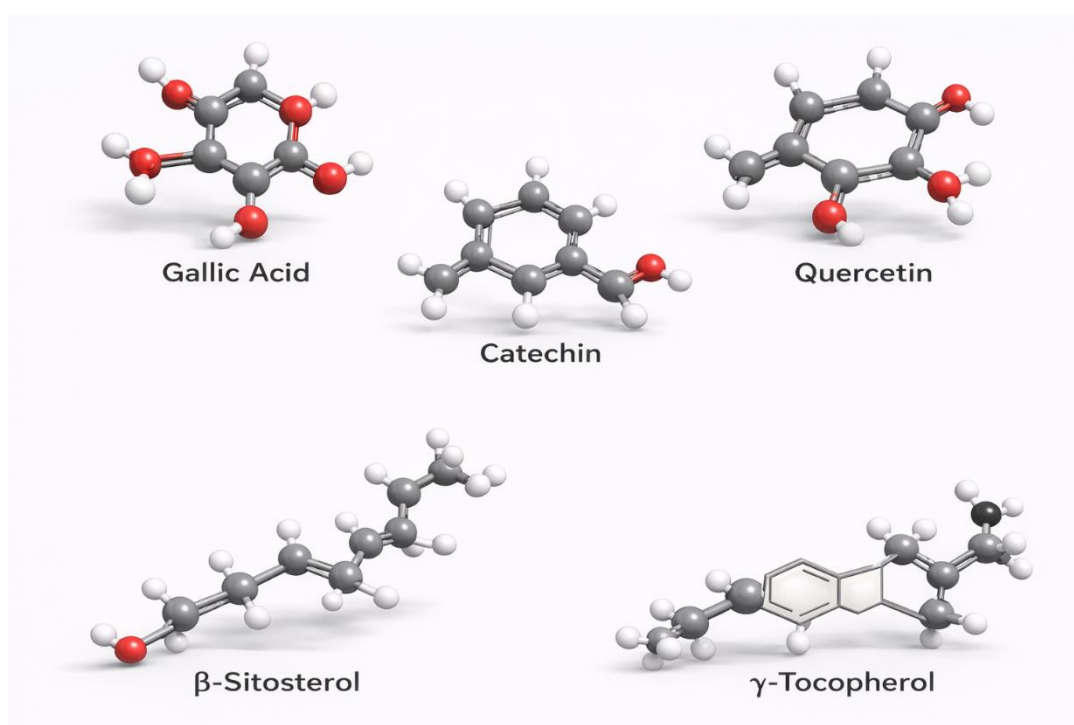


Figure 11. The main bioactive component in Carob.

Source:(Hadi et al., 2017)

Table 5. Applications of Carob in different biotechnological processes

Biotechnological Process	Carob Application	Specifics	References
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Fermentation	Utilized carob in fermentation processes	Carob-derived sugars serve as a carbon source for microbial fermentation, enhancing the growth and metabolic activity of probiotic bacteria.	Aboura et al., 2017
Bioethanol Production	Contribution of carob in bioethanol production	Carob pods contain high fermentable sugar content, making them a suitable substrate for bioethanol production.	López-Sánchez et al., 2018
Enzyme Production	Application of carob in the production of specific enzymes	Carob-derived substrates support the production of hydrolytic enzymes (e.g., cellulases, pectinases, and xylanases) used in food and feed industries	Fidan et al., 2020
Bioremediation	Use of carob for bioremediation purposes	Carob polyphenols and fibers contribute to soil detoxification and heavy metal absorption, improving soil health and microbial diversity.	Goulas et al., 2016
Probiotic Growth Medium	Carob as a natural prebiotic	Carob fiber and galactomannans enhance the growth of beneficial gut microbiota, supporting probiotic formulations.	Krokou et al., 2019
Food Preservation	Carob-based natural preservatives	Carob extracts exhibit antimicrobial and antioxidant activity, prolonging the shelf life of perishable food products.	Özcan et al., 2009

Citric Acid Production	Carob as a substrate for <i>Aspergillus niger</i> fermentation	High sugar content in carob pods supports citric acid biosynthesis, used in the food and pharmaceutical industries.	López-Sánchez et al., 2018
Biopolymer Production	Extraction of locust bean gum (LBG) from carob seeds	Carob seeds provide LBG (thickener E410), used in food, cosmetics, and pharmaceutical formulations	Matthäus & Özcan, 2011
Functional Food Development	Carob-based functional foods	Carob is incorporated into high-fiber, gluten-free, and antioxidant-rich food formulations.	Tounsi et al., 2017
Prebiotic & Dietary Fiber Production	Carob-derived galactomannans	Galactomannans extracted from carob seeds serve as a dietary fiber source with prebiotic properties.	Fidan et al., 2020
Chocolate Alternative	Carob as a cacao substitute	Used in chocolate-free confectionery, ideal for individuals with caffeine sensitivity or chocolate allergies	Özcan et al., 2009

Chapter 3.
Biotechnological and health
Benefits of Soybean
(Glycine max)

1. Soybean: Chemical Profile and Biotechnological Potential

1.1. Introduction to *Glycine max*

Soy (*Glycine max*) is a leguminous crop originating from East Asia and occupies an important position in world agriculture due to its high protein, adaptability, and a wide range of uses. The cultivation of soybeans has since expanded to multiple climate zones worldwide, with around 85% of the crop grown in the United States, Brazil, and Argentina, and it is considered a major source of plant-based protein and healthy fats. Soybeans also possess tremendous versatility as food and non-food products. Soy offers other uses outside of the food industry- in fact, it's used to produce adhesives, plastics, biofuels, and compostable materials, as well as demonstrating their importance to any environmentally conscious market (Junaidi et al., 2025; L. Liu et al., 2022; Rizzo, 2024). Despite their decades-old cultivation and use, attempts toward researching and developing the potential applications of the soybean itself in terms of functional food, biotechnology, sustainable agriculture, etc., are still going on to some extent. The increasing integration of soybeans into plant-based diets, meat substitutes, eco-friendly products, and various passive uses speaks to their ever-growing importance within the food and industrial landscapes at a global scale.(Rizzo, 2024). .

1.2. Botanical classification

- Kingdom: *Plantae* (Plants)
- Division: *Streptophyta*
- Class: *Equisetopsida*
- Order : *Fabales*
- Family: *Fabaceae* (Legume family)
- Genus: *Glycine*
- Species: *Glycine max*

1.3. Geographical distribution according to (POWO, 2025; The Plant List., 2023)



Figure 12. Aspect of a Soybean Sample

Glycine max, referred to as Soybean, is native to the Asian regions. Gradually, it was spread to other regions with conditions, including North Africa, the Middle East. (For a clearer perspective of its spreading, refer to Figure 11.)

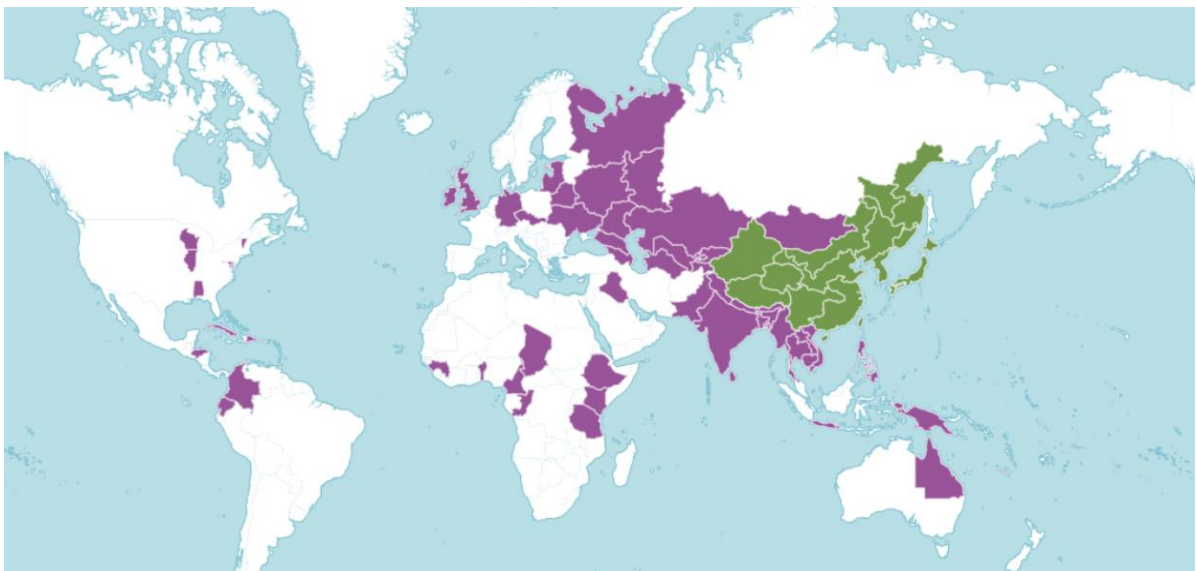


Figure 13. Distribution of Beta vulgaris Worldwide: Green: Native / Violet : introduced to

1.4. Soybean Cultivation Regions in Algeria

Soybean (*Glycine max*) cultivation in Algeria has been expanding, particularly in areas suitable for its growth. Prominent regions include:

1. **Bordj Bou Arréridj:** Situated in the north-central part of Algeria.
2. **Setif:** Located in the northeastern part of the country.
3. **M'sila:** In central Algeria, M'sila is involved in various agricultural projects.
4. **Laghouat:** Situated in the southern part of Algeria.
5. **El Bayadh** – Located in the western part of Algeria



Figure 14. Distribution map illustrating the five regions in Algeria recognized for soybean cultivation: Bordj Bou Arréridj, Setif, M'sila, Laghouat, and El Bayadh

1.5. Chemical composition of Soybean

Soybean seeds contain approximately 20% oil, 40% protein, 35% carbohydrate, 5% ash, and 1% moisture (Rojas-Beltran & Narro-Sanchez, 2003). In some cases, protein content may reach 45%, especially in seeds produced at higher latitudes during cooler seasons; oil content can range from 15% to 25%. Percentages of carbohydrates can also be in the range of about 25% to about 40%. Proteins and non-protein nitrogen compounds of soyfoods in the development of beany flavor. Soybean threonine, cystine, isoleucine, and tyrosine values are much greater than those of egg, milk, or meat proteins (Ayu et al., 2023). The Oils are the glycerol esters of fatty acids. The triglyceride fraction of the seed is the major energy source for germination and growth of cotyledonary seedlings (Talarico & Dobrogosz, 1989). An analysis of 200 samples from three growing regions and 20 US soybean varieties showed average linolenic, linoleic, oleic, and palmitic acid of 2.21–6.56%, 44.8–52.7%, 19.8–29.4% and 10.1–11.9%, respectively (Sanchez et al., 2006). Excessive amounts of linolenic acid are not desired, since it makes the oil become rancid (oxidize) over time in the storage of the germ and crude oil extracted.

1.5.1. Key Compounds Highlighted

Phospholipids are the most abundant in linolenic acid and have slightly more linoleic and slightly less oleic acid than total soybean oils. Phosphatidylcholine - The Major Phospholipid of Soy About a fifth (20%) of soybean phospholipids is made up of soy lecithin's major phospholipid (Ayu et al., 2023). Lecithin exhibits both oil-in-water (hydrophilic) and water-in-oil (lipophilic) properties in emulsion formulation and has been used in the food industry. It is also surface active, which helps in emulsification and inhibits proteins from sticking to the metal surface. Lipid-lowering effects have been described for phosphatidylcholine as a dietary supplement. This could be from inhibiting micelle formation in bile salts (Rizzo, 2024).

Phosphatidylethanolamine (PE) constitutes around 15% of phospholipids, has been implicated in membrane fusion, and can produce pores in membranes. Soy is the only vegetable protein that contains sufficient quantities of the sulfur-containing amino acids methionine and cysteine. Given that cysteine is subject to oxidation, it may be formed into a disulfide bond between different cysteines (the ratio of the two can vary widely among proteins) (Elhalis et al., 2024).

1.6. Bioactive Components of Soybean

Soybeans (*Glycine max*) are a rich protein source and contain other bioactive compounds. Among these, isoflavones such as genistein, daidzein, and glycitein are the most studied. As phytoestrogens, these isoflavones mimic estrogen's effects in the body, particularly supporting hormonal balance in postmenopausal women (Wei et al., 2020). Soybeans are also rich in saponins and phytosterols, especially beta-sitosterol, which help reduce cholesterol and support heart health (Pawłowska et al., 2018). Additional bioactive compounds include peptides from protein hydrolysis, which have antihypertensive and antioxidant effects. The majority of studies on isoflavones and lignans have been in relation to hormone-dependent cancers and coronary heart disease (Kim et al., 2004). Approximately 30-50mg of isoflavones are contained in soy products per 100g, with the majority coming from glycosides daidzin, genistin, and glycitin. These isoflavones can have differing physiological effects. For example, genistein can increase serum cholesterol, whereas daidzein can help reduce it. Isoflavones are also known to decrease LDL oxidation, which is one of the initial steps in atherogenesis (Chiou & Cheng, 2001).

1.6.1. Other relevant substances

Saponins and lignans are two major classes of substances contained in soybeans and are situated largely in the soybean skins. Saponins have been shown to be able to lower the elevated blood lipids and the risk of cardiovascular diseases through their effects on lipid metabolism. While lignans have natural antioxidant properties and can reduce cardiovascular diseases along with some anti-cancer effects, though results are inconsistent (Mikołajczyk-Bator et al., 2016).

Phytosterols are a minor constituent in soybeans, but their consumption has been shown to have hypocholesterolemic effects throughout various studies. Soy products with enhanced levels of phytosterols can be an important future health food.

1.7. Health Benefits Associated with Soybeans

1.7.1. Cardiovascular Health

High consumption of soy food has been found to correspond with a low risk of CHD and other CVD. CHD and stroke are the leading killers in most countries, caused by the formation of plaque in arteries that eventually get blocked or burst. All this plaque develops because of high levels of LDL cholesterol and oxidation of the same cholesterol. A meta-analysis of 38 clinical

studies recently found that consuming soy protein with isoflavones decreased LDL cholesterol by 12.6 milligrams per deciliter compared to milk or other proteins. Another study found that consuming two veggie burgers a day with 25g of soy protein significantly decreased LDL cholesterol by 9% after just three months. This immense lowering of LDL cholesterol has been proposed as the leading way that soy consumption prevents heart disease; for each 1% a person's LDL level declines, his/her risk of heart attack correspondingly drops by 2%. Prevention of Heart Diseases isoflavones, saponins, and tocopherols have been consistently associated with various health benefits, and a direct link for their effect on cardiovascular health is yet to be well understood.

1.7.2. Lowering Cholesterol Levels

Soy protein has the potential to be supported as a natural approach to reducing cholesterol. The US Food and Drug Administration announced on October 26, 1999, that there is "strong" evidence for allowing health claims on labels of soy protein foods being able to reduce the risk of CHD(FDA, 2019). When individual study results were pooled together, 38 controlled clinical studies showed an average reduction of 9.3% total blood cholesterol and a decrease of LDL cholesterol of 12.9 mg/dl(Eslami & Shidfar, 2019).

1.7.3. Reducing Blood Pressure

High blood pressure (hypertension) is a long term medical condition in which the blood pressure is so high that it puts excessive strain on the arteries. In this meta-analysis of 23 randomized trials, soy protein reduced SBP by 2.21 mmHg and DBP by 1.44 mmHg compared to a casein diet. A drop in blood pressure was significant for all the subgrouping factors of various types of patients(SU et al., 2018). The greater differences of these results occurred when using high blood pressure subjects, with average reductions for systolic and diastolic of 7.58 and 5.48 mm Hg respectively.

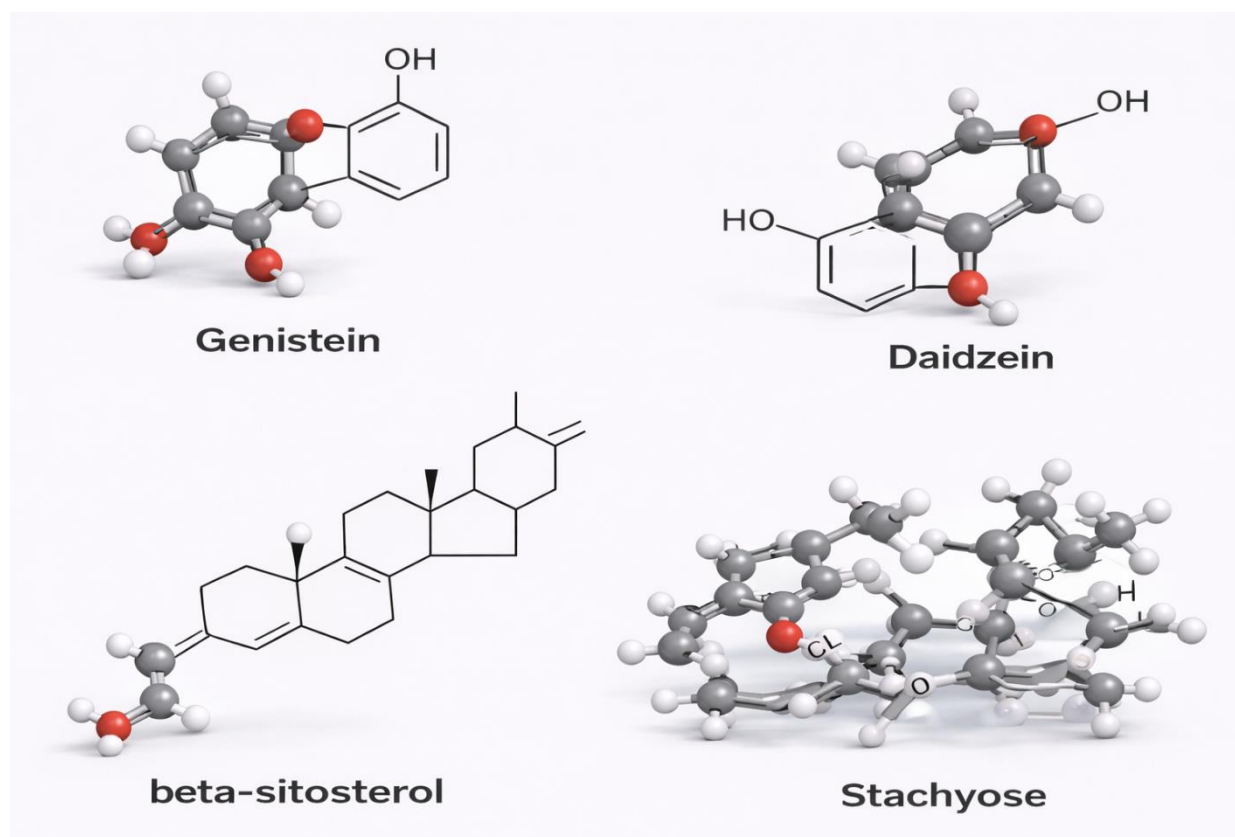


Figure 15. Molecular structures of key bioactive compounds in soybean.

1.8. Biotechnological usage of Soybean

Soybean (*Glycine max*) plays a significant role in biotechnology, being utilized both for the production of various biomolecules and for agricultural and nutritional improvements. On one hand, due to its abundant biomass and high protein content, the plant has been used as a "plant bioreactor" for the expression of recombinant proteins for pharmaceutical and industrial purposes (Vianna et al., 2011).

On the other hand, soybean is a model for genetic engineering and marker-assisted selection to improve traits such as resistance to pathogens, adaptability to abiotic stresses, and the lipid composition of soybean oil (Zhu et al., 2023). Specifically, in the context of healthier food sectors, biotechnologically improved soybean cultivars have been developed using genetic engineering approaches to obtain oils with higher oleic acid content and reduced saturated fatty acids, thus improving the oxidative stability and nutritional profile of the final product (Kang et

al., 2023). These innovations position soybean as a multifunctional platform that aligns with sustainability and the valorization of plant resources (Wei et al., 2020).

1.8.1. Applications in the Food and Agricultural Industries

In the food sector, LAB-fermented soy products are popular due to their rich protein content, probiotic effects on digestion, and use as useful ingredients in vegetarian meals. Fermented soy proteins, in particular, are becoming increasingly popular meat replacers with significant potential for sustainable and nutritious plant-based diets. The integration of LAB fermentation into soy-based food systems results not only in a nutritionally valuable product, however in improved flavour, texture, and digestibility, providing a more desirable targeted for traditional and modern food markets (Elhalis et al., 2024). The improvement of protein bioavailability is one of the main advantages of LAB fermentation of soybeans. Proteins from soybean, despite possessing high levels of essential amino acids, are frequently regarded as indigestible because they contain anti-nutritional factors such as protease inhibitors (Huang et al., 2023). LAB fermentation helps reduce these things by taking the soybean proteins and breaking them down into smaller peptides and free amino acids that are more easily utilizable by our body. Certain LAB strains, e.g., *L. acidophilus* and *L. rhamnosus*, produce proteolytic enzymes that degrade these inhibitory compounds, leading to an increase digestibility of proteins (Elhalis et al., 2024; Huang et al., 2023).

LAB fermentation influences fiber digestibility, besides improving isoflavone bioavailability. Soybeans are rich in soluble as well as insoluble fibers, which are important for gut health (Gan et al., 2023; Panchal & Patel, 2014). LAB ferments these fibers to produce short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, which can provide energy for the beneficial microbiota and play an important role in intestinal health promotion by preserving mucosal inflammation and regulating gut homeostasis (Zhao et al., 2020). Increased production of SCFAs has not only a beneficial effect on digestion but also systemic effects such as anti-inflammatory and immunomodulating (Kim et al., 2018).

The LAB fermentation of soybeans increases their nutritional value by increasing the bioavailability of proteins, minerals, and bioactive compounds, as well as reducing anti-nutritional factors, including protease inhibitors and phytates, leading to better absorption of essential amino acids and minerals (Chiou & Cheng, 2001). Furthermore, LAB fermentation generates bioactive

peptides and short-chain fatty acids (SCFAs) that contribute to gut health, lower the risk of cardiovascular diseases, and stimulate immune system response (Kim et al., 2016; SU et al., 2018).

1.8.2. Soybean in Cosmetic and Pharmaceutical Applications

processes in several sectors for their bioactive molecules. In the pharmaceutical industry, LAB fermented soybeans provide a high source of bioactive components with potential therapeutic benefits for illnesses including inflammation, cancer, and cardiovascular disease. The fermentation of soy increases the bioavailability of isoflavones and other antioxidants that have been used for anti-inflammatory and anticancer effects. LAB-fermented soy products have become increasingly popular in the market of nutraceuticals (Sirtori, 2001; Zhao et al., 2007). where natural health products are demanded/required as a sustainable and effective substitute for synthetic drugs.

Extracts of fermented soy, rich in isoflavones with various biological activities (antiaging, anti-inflammatory, and moisturizing), are popular ingredients for cosmetics. These bioactive ingredients protect the skin from oxidative stress, increase elasticity, and reduce the appearance of wrinkles. Since, LAB-fermented soy product also possesses multifunctionality for use in natural cosmetic products that aim to maintain the skin's look young and healthy(Canter et al., 2005).

Soy lecithin decreases cholesterol levels and contributes to fighting against chronic liver diseases (Edziri et al., 2019). In addition, soy (especially genistein)-derived isoflavones could prevent bone loss and decrease the incidence of osteoporosis by exerting estrogen-like activity. Frequent consumption of soy-based products decreases your chance of developing heart disease by increasing flexibility in the blood vessels and preventing plaque buildup in the arteries (Kim et al., 2004).

1.8.3. Soybean in Culinary Usages

Traditional fermented soybean products are becoming an increasingly important component of the human diet. The extracellular enzymes from microorganisms could catalyze biochemical reactions of soybean nutrients during the fermentation. In this way, bioactive compounds such as bioactive peptides are generated (Liu et al., 2022). Soybeans fermented by LAB are also useful for the agricultural field, not only in sustainable agriculture. The LAB-fermentation can enhance the quality of soil, and it can also offer biocontrol possibilities for a reduction in chemical pesticides. Furthermore, fermented soy products may be used in the

production of bio-based fertilizers to ensure a more sustainable agriculture. These emphasize the significance of LAB fermentation not only in the food and health sector, but also in improving environmental sustainability, and development of ecologically sustainable agricultural technologies (Alshannaq & Yu, 2017; Ikram et al., 2023; L. Liu et al., 2022)

Soy products, including tofu, soy milk, tempeh, and soy protein isolates, have become particularly popular as substitutes for animal-based products due to the availability of options that can mimic equivalent texture, taste, and nutritional components (Rizzo, 2024). These foodstuffs are of particular importance in vegetarian and vegan diets, and they are increasingly being acknowledged for their role in cardiovascular disease prevention, cholesterol-lowering effect, and bone health (Zhao, 2007).

Table 6. Examples of Soy-based Foods

Soybean-based Food	Characteristics	References
Tofu	Rich in protein, low in fat, high in calcium, versatile for savory or sweet dishes	(Mateos-Aparicio et al., 2008)
Soy Milk	Lactose-free, high in protein, calcium, and vitamins B and D	(Eslami & Shidfar, 2019)
Tempeh	Fermented soy product, high in protein, probiotics, and vitamins	(Rizzo, 2024)

Chapter 4

Biotechnological involvement
of Lactic Acid Bacteria
in Plants Valorisation

1. Introduction

The lactic acid bacteria (LAB) comprise a diverse group of Gram-positive non-spore-forming microaerophilic or facultatively anaerobic cocci or rods. They are important microorganisms that are widely used in making fermented foodstuffs like cheese, yogurt, pickles, beer, wine, cider, sauerkraut, and cured meats in this laboratory (Microbiological Encyclopedia, 2010). As LAB are significant members in the food industry, the determination of the nutritional requirements of these bacteria has been studied extensively. We will answer such questions in this essay based on knowledge and evidence from many scientific articles.

Lactic acid bacteria (LAB) were first discovered and employed in food processes at the same time as mankind started fermenting vegetables, fruits, milk, and grain into pickled products. The history of fermenting food products is suggested to be witnessed when people left their food in a cold, dark place, and it became pickled (Saris, 2014; Stiles and Holzapfel, 1997). It was the first way of food preservation that is still practiced in third-world countries today. It was one way in which food could be preserved and kept for hard times of famine and long winters.

LABs are believed to have appeared shortly after 4000 BC in Babylon, where they were employed in beer production. Long before the concepts of microbes and fermentation, they merely understood that if a couple of days of contact with the environment were given to barley and water, the result was an intoxicating drink (Schillinger & Holzapfel, 2003). The modern history of LAB really goes back only 330 years to when a Dutchman, Leeuwenhoek, first observed and described, in 1684, the action of fermentation. This was the beginning of microorganisms, and he coined the term "wee animalcules" to describe them. And since no one knew what fermentation even was for another 200 years, little research progressed on LAB and their fermentations (Saris, 2014; Stiles and Holzapfel, 1997).

1.1. Importance of Lactic Acid Bacteria in the Food Industry

The process of conversion of milk to different fermented products is considered to be as old as 6,000 years. Since then, there has always been uncertainty about the exact nature of fermentation and the types of microorganisms involved. It wasn't until the mid-19th century that Louis Pasteur demonstrated the connection between the spoilage of wines or beer and the activities

of microorganisms. Two types of fermentations were categorized: one being the spoilage of produce, wherein off-odors, flavors, and slime are produced, and the other being fermentation, where energy-yielding reactions of carbohydrates end up with an organic molecule. The final product of this pyruvate dictates the type of fermentation that has occurred.

In modern times, lactic acid bacteria (LAB) have become central figures in the industry of fermented foods due to the knowledge of their metabolic functions. They have been isolated and used for the production of foods such as yogurts, cheeses, fermented meats, and vegetables. The typical genera of LAB involved in these processes are *Lactobacillus*, *Streptococcus* (now known as *Lactococcus*), and *Bifidobacterium*. These organisms displace the original microflora of the food substrate through competition and production of inhibitory substances, which prevent spoilage and pathogens from growing. The LAB can alter the texture and flavor of many different food types (Seddik et al., 2017). For example, in yogurt, the thickening of the milk is due to exopolysaccharides synthesized from lactose by bacterial β -galactosidases. LAB fermented foods have become increasingly popular in diets due to the associated health benefits. For example, yogurt prevents lactose intolerance due to the consumption of lactose, and it is believed that probiotics from fermented foods have a positive impact on the microflora of the lower intestinal tract (Gómez-Torres et al., 2016). The use of LAB is not just limited to the fermentation of dairy products, but rather a large range of foodstuffs. It would be hard to find a foodstuff that hasn't been subject to lactic acid fermentation.

1.2. physiology

Lactic acid bacteria (LAB) are largely defined by their capacity to ferment carbohydrates, namely sugars, into lactic acid, which is essential in the fermentation of food. They are facultative anaerobic, growing in the presence or absence of oxygen. LAB metabolize sugar via glycolysis, leading primarily to lactic acid, but some species will produce additional metabolites such as acetic acid or ethanol. This metabolic process is important for food preservation when the lactic acid decreases the pH and prevents the growth of spoilage microorganisms.

LAB are very well suited to survival under difficult conditions, for example, in the acid environment of the stomach or during fermentation under low oxygen conditions. They have peptidoglycan in cell walls for structural protection, have different transport systems for smaller

and larger molecules, and have different stress-defense systems. These characteristic physiological properties allow LAB to exist in various environments and act as probiotics, serving to be beneficial for gut health, immune function, and pathogen inhibition.

1.3. Nutritional Requirements of Lactic Acid Bacteria

LAB are important in the food industry and are generally regarded as safe (GRAS) organisms that help ferment edible foods and feed substrates by causing desirable modifications resulting from the production of organic acids and bacteriocins (Sadiq et al., 2019). They possess the ability to metabolize a wide variety of carbohydrates, which allows them to be successful in multiple environments and efficiently colonize and compete with other bacteria. Although the carbohydrate metabolism and genetics of lactobacilli have been studied extensively, relatively little is known about their amino acid and protein metabolism, which is somewhat surprising in view of the fact that dairy-based substrates such as milk whey are poor in amino acids and neutral nitrogenous compounds.

Most LAB need a variety of nutrients such as B vitamins and amino acids (as reported by Kitay & Snell, 1950; Snell, 1945), but, as pointed out by Tannock *et al.* It highlights the need for a better comprehension of their nutritional requirements, not only to ameliorate fermentation processes, but also to further understand them as microbial agents in different ecological niches. Earlier reports by Orla-Jensen (1919, 1930) had already pointed out the need for complex media for high growth rates of LAB, recognizing some important components including riboflavin and individual amino acids; however, most LAB still require culture on undefined nutrient sources, which indicates a persisting drawback in this field of research (ROGOSA et al., 1947; TITSLER et al., 1947).

LAB media, especially those used for food fermentation, must have a delicate balance of the growth nutrients, including nitrogen sources, carbon sources, and the buffer system. The most commonly used de Man, Rogosa and Sharp (MRS) medium offers LAB a standardized growth environment, but it is expensive mainly due to the nitrogen sources (ROGOSA et al., 1947). The nitrogen sources in these media are generally expensive and contribute, to a large extent, to the cost of culture medium. This has prompted a continuing search for alternative, inexpensive media. Some research work has been undertaken highlighting the possibility of substituting expensive nitrogen sources in LAB culture media with cheaper components obtained from agronomic by-

products, i.e., wheat bran hydrolysates and steep corn liquor. These options not only save costs, but also can make waste resources recycled, which is helpful for food industry sustainable production methods (Zhao et al., 2019).

1.4. Carbohydrates as a Source of Energy

In the case of many fermentable pentose sugars as well as glucose, an alternative phosphor-ketolase pathway allows the metabolism of sugar phosphate to produce energy with lactic acid and/or ethanol end products, where 1 mole of sugar phosphate can be split to yield 1 mole of lactic acid and 1 ATP (Vuyst et al., 1993). This pathway is exhibited by *L. plantarum* in the metabolism of ribose. High energy yield from hexose and pentose sugar phosphate may create a need for increased reoxidation of NADH, and it has been suggested that sugar fermentation by LAB is linked to a specific sugar-inducible NADH-dependent lactic acid dehydrogenase, to optimize lactic acid production in comparison to mixed acid fermentations (Baygut et al., 2023). The first and rate-limiting step in the catabolism of hexose sugars occurs with the formation of sugar phosphate via the action of sugar-specific phosphoenolpyruvate: sugar phosphotransferase system (PEP-PTS).

Subsequent conversion of sugar phosphate to pyruvate or lactic acid with ATP production occurs by a common Embden-Meyerhoff pathway. This pathway has been described in detail for *lactococci* and *Lact. helveticus* when using glucose, and it was shown that a high ATP yield of 2 moles per mole of sugar fermented occurs during lactic acid production from sugar phosphate by pyruvate kinase action. This compares with a 1 ATP yield via pyruvate formation and 2 moles of lactate dehydrogenase reduced pyruvate to lactic acid with a net energy yield of 1 mole of ATP and 1 mole of lactic acid. Control of lactic acid production varies among different LAB and is influenced by the metabolic requirement for NADH reoxidation to regenerate NAD⁺ (Djeghri-Hocine et al., 2007; Valerio et al., 2008; Yoon et al., 2004).

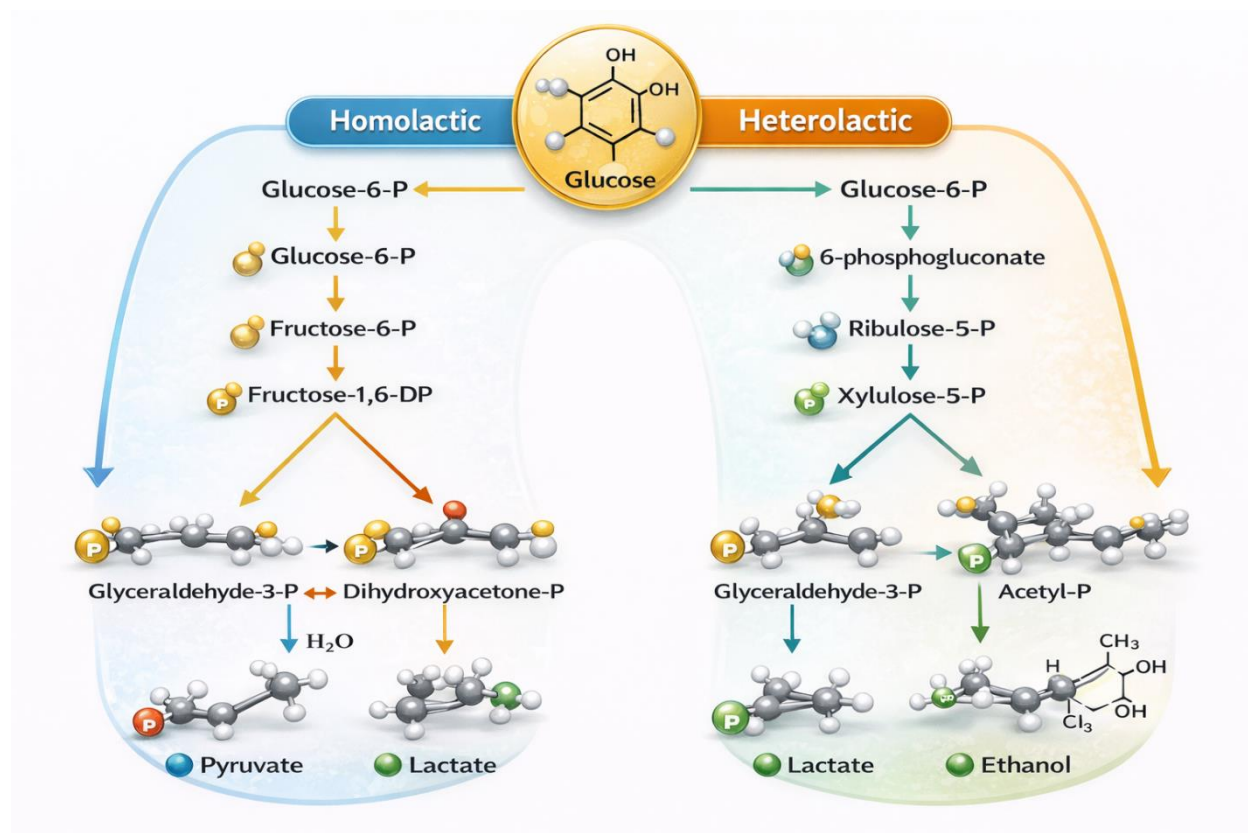


Figure 16. Generalized scheme for the fermentation of glucose in lactic acid bacteria(Caplice, 1999)

Lactobacillus plantarum and *L. casei*, typical of heterofermentative lactic acid bacteria (LAB), exhibit environmental versatility by utilizing a wide range of carbon sources. For these microorganisms, carbohydrates must undergo catabolism to release metabolic energy, with the primary interest being lactic acid formation. Energy production is measured in terms of ATP, and studies using ^{14}C -labeled substrates have quantified carbon recovery as CO_2 and lactic acid. ATP yield is generally in a 1:1 ratio with the sugar consumed, except for galactose, which is fermented to pyruvate and lactic acid at the expense of one ATP. Holzapfel et al. (1990) confirmed a 1:1 ATP-to-glucose ratio for *L. casei* and other sugars, supporting the idea that sugar fermentation rate is primarily driven by energy needs rather than just acid production.(Stiles and Holzapfel, 1997)

1.5. Proteins and Amino Acids for Growth

1.5.1. Essential Amino Acids for Lactic Acid Bacteria Growth

There are 20 amino acids required for protein synthesis in all organisms. Ten of these are synthesized by the transamination and deamination of their keto acids. The remaining ten have to be provided in the growth medium since the bacteria do not possess all the enzymes required for their biosynthesis. The keto acids of the essential amino acids can only replace the amino acids themselves if there are other amino acids present in the medium that allow the synthesis of that keto acid. The availability of these amino acids in the medium alters the metabolic activity of the bacteria and has drastic effects on the growth and production of organic compounds.

1.5.2. Leucine

Leucine increases biomass yields of lactic acid bacteria, with the extent to which it does this varying between the different species. In a study looking at the effect leucine has on different strains of *Lactobacilli*, it was found that *L. casei subsp. Lactobacillus rhamnosus* was the most stimulated by leucine, with biomass yields increasing 6.1-fold compared with the control. In contrast, leucine did not affect *L. delbrueckii subsp. bulgaricus*. Despite this, *Lactococcus lactis subsp. Cremoris* has been found to require leucine for growth. In the latter study, it was identified that leucine was first in a sequence of four enzymes catalyzing the biosynthesis of branched-chain fatty acids required for the plasma membrane in *Lactococci*. The importance of these fatty acids has been displayed in a study, which showed that mixed isomer unsaturated C(18) fatty acids

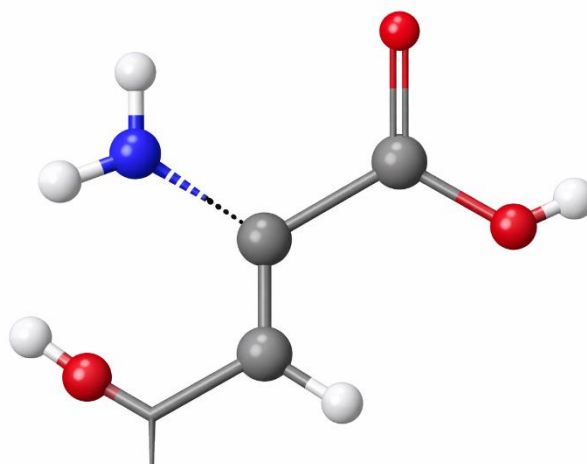


Figure 17.Leucine : (2S)-2-amino-4-methylpentanoic acid retrieved from PUBCHEM CID 6106

effectively increased plasma membrane fluidity and protected cells against cold stress (Zhang, 2019)

1.5.3. Valine

Valine is a relatively apolar and is one of the essential amino acids. It is unique in that it can act as both a hydrophobic and a hydrophilic substance due to the way it is able to fit within a protein structure. This means that valine is taken into the cell by a single transport system. This system is wasteful and a little nonspecific due to the fact that this one transport system must be capable of distinguishing between all three leucine, isoleucine, and valine to select proline.

Although valine can be synthesized into prothorone, it is not able to take the place of isoleucine or leucine in the synthesis of cell components, and thus valine is considered to be essential with respect to that role. In terms of what valine actually does for bacteria and why it is essential in its role, there is very little evidence to suggest its specific functions, and it has been described as a "functionally silent molecule." While studying the effects of valine-starved mutants of *E. coli* by using a variant RNA stable isotope labeling method, it was shown that the destruction of rRNA was affected during valine starvation. Although we are only on the brink of understanding the specific roles of amino acids at the molecular level, it is likely that valine no doubt plays a role in the stability of genetic material and the maintenance of appropriate protein folding (Jiang et al., 2020)

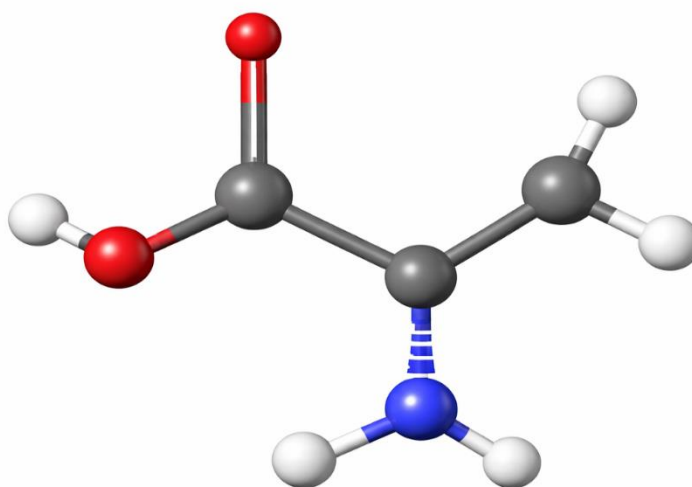


Figure 18. Valine (2S)-2-amino-3-methylbutanoic acid retrieved from PUBCHEM
CID 6287

1.5.4. Isoleucine

Isoleucine is one of the essential branched-chain amino acids (BCAAs) that play a vital role in various metabolic processes, including protein synthesis, energy production, and muscle repair.

As an essential amino acid, isoleucine cannot be synthesized by the human body and must be obtained through dietary sources such as meat, dairy, and legumes. It is involved in maintaining blood sugar levels by stimulating glucose uptake into cells, particularly in muscle tissue, during exercise (Krämer et al., 2020). Isoleucine also serves as a precursor for various metabolites and plays a crucial role in the regulation of immune functions and hemoglobin production (Li et al.,

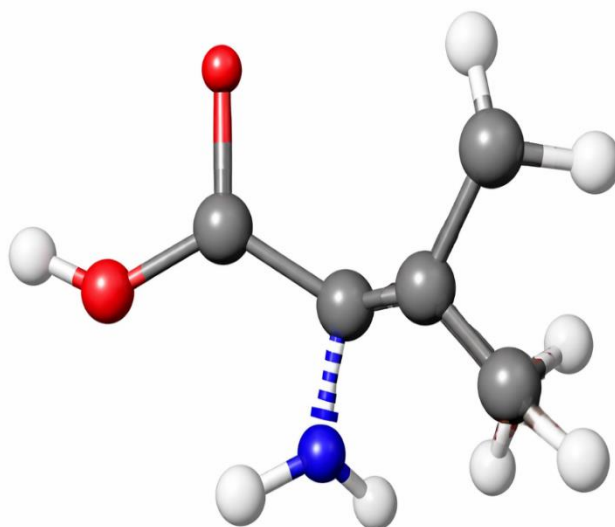


Figure 19. Isoleucine (2S,3S)-2-amino-3-methylpentanoic acid retrieved from

1.5.5. Lysine

Lysine is an essential amino acid for several lactic acid bacteria, including *Bifidobacterium acidilactici* and *Pediococcus acidilactici*, which require it for growth. In *Bacillus subtilis*, lysine serves as a precursor for cell wall synthesis, with diaminopimelate epimerase being a key enzyme in its biosynthesis pathway (Tzin & Galili, 2010). This enzyme is regulated by a lysine repressor, which downregulates transcription of genes involved in lysine biosynthesis when intracellular

lysine is in excess (Berlowska et al., 2016). This is evident in *Lactobacillus* species, where the absence of lysine or diaminopimelate epimerase leads to a hairless mutant phenotype due to impaired cell wall synthesis. Several *Lactobacillus* species, such as *L. plantarum*, *L. helveticus*, and *L. salivarius*, have been shown to exhibit lysine auxotrophy, making lysine a required growth factor.

1.5.6. Methionine

Methionine, the only sulfur-containing amino acid, plays a crucial role in the growth and metabolic activity of *Lactobacillus bulgaricus*. This microorganism produces enzymes that degrade excess *L-methionine* into α -keto acids and various carboxylic acids, particularly when L-methionine concentration exceeds 0.5% of the dry cell weight. At this level, cell growth becomes insignificant, with only small amounts of L-methionine being used for protein synthesis. Conversely, a deficiency of *L-methionine* impairs the growth of *L. bulgaricus*, a fastidious microorganism that is auxotrophic for several amino acids. L-methionine is essential not only for protein synthesis but also as a precursor for important carboxylic acids and for the production of enzymes that enhance gene expression.

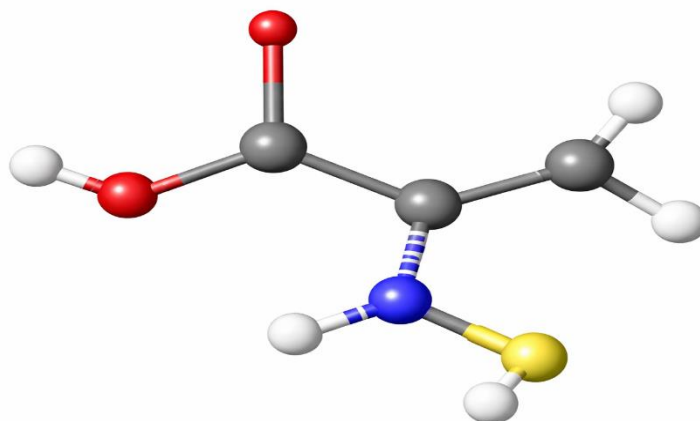


Figure 20. Methionine (2S)-2-amino-4-methylsulfanylbutanoic acid retrieved from PUBCHEM CID 6137.

1.6. Non-Essential Amino Acids for Lactic Acid Bacteria Growth

Amino acid supplementation, including alanine, glycine, and glutamate, to *Lactobacillus delbrueckii subsp. Bulgaricus* showed a significant impact on its growth and acidifying activity. Glycine was non-essential, but alanine and glutamate were essential for PS140 T. The bacteria seemed to require alanine and glutamate, as they produced no growth with either. *L. bulgaricus* can metabolize exogenous glutamate, an amino acid acting as a metabolic precursor for some other biological molecules. Peptides released during milk protein hydrolysis that occurred during fermentation also stimulated bacterial growth, which suggests that *L. bulgaricus* might prefer to utilize this rather than alanine. These results may have implications for stimulating bacterial growth in industrial fermentations used in food production

1.6.1. Alanine

The growth of *Lactococcus lactis* cells is stimulated by alanine, resulting in reduced generation time and increased final cell density. However, alanine also acts as an "exhaustion" substrate in *L. casei* during chemostat cultivation, eventually leading to cell autolysis. Recent studies have clarified the biochemical pathways and regulatory mechanisms behind alanine's effect on cell growth. In *L. casei*, aminotransferase activity with 2-oxoglutarate was significantly higher in cells grown with alanine, indicating that alanine enhances the synthesis of 2-oxoglutarate. Alanine is catabolized through transamination with 2-oxoglutarate to produce pyruvate and 2-oxoglutarate. The increased aminotransferase activity and carbon flow regulation suggest that alanine can induce carbon catabolite repression (CCR), affecting the growth of lactic acid.

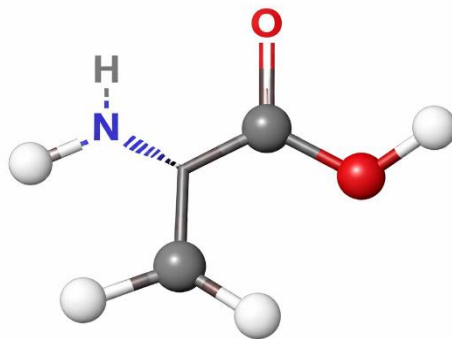


Figure 21. Alanine (2S)-2-aminopropanoic acid retrieved from PUBCHEM CID 5950.

1.6.2. Glutamate

Glutamate serves as a critical nitrogen source for asparagine biosynthesis, as demonstrated by mutations in glutamine synthetase, which regulate the GOGAT pathway based on available nitrogen sources. This mutation allows the study of nitrogen mobility and inter-enzyme product transfer between reactions in the alternative and original biosynthetic pathways. For example, the formation of an isozyme of GOGAT, which bypasses normal regulation by the nitrogen source, enables direct ammonia donation to produce glutamate from 2-oxoglutarate, preventing the catabolism of accumulated glutamine (Dallagnol et al., 2011).

The presence of pyridoxal in the medium was essential for the growth of *Lactobacillus casei* and other pyridoxal-dependent LAB when glutamate was the nitrogen source, highlighting the importance of transamination in glutamate metabolism. In contrast, growth was not observed when glutamate was the sole nitrogen source, suggesting that the synthesis of glutamine from exogenous glutamate is necessary for *L. casei* growth. Additionally, growth in a medium containing ammonium chloride as the nitrogen source was inhibited by higher glutamate concentrations, indicating feedback inhibition of glutamine biosynthesis by glutamate (Gamage et al., 2016).

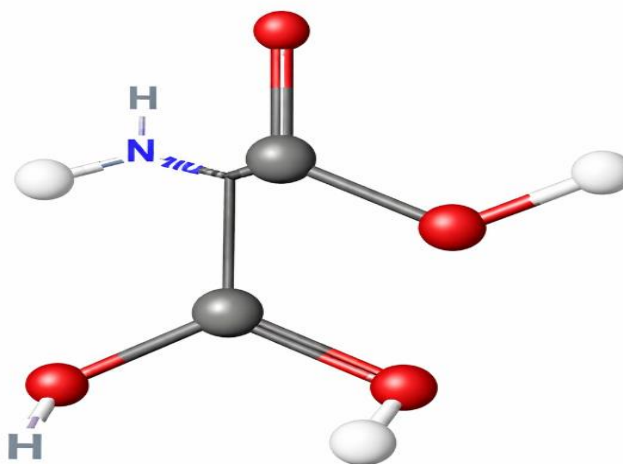


Figure 22. Glutamate (2S)-2-aminopentanedioic acid retrieved from PUBCHEM CID 33032.

1.6.3. Glycine

Glycine is a copious and metabolically flexible amino acid which, although not essential in the human diet, is indispensable for the growth of diverse microorganisms, including lactic acid bacteria. Junaidi et al. (2025) reported that glycine was the sole amino acid required for the growth of *Lactobacillus* species (*L. arabinosus*, *L. casei*, and *L. plantarum*) in a defined medium containing basal salts, organic acids, inulin, vitamins, and trace minerals (Junaidi et al., 2025). Glycine's low molecular weight makes it one of the smallest amino acids, with side chains consisting solely of a hydrogen atom (although evidence suggests that it can freely rotate). Its uses range from serving as an inhibitory neurotransmitter and neuromodulator to being used as a precursor to the biosynthesis of proteins and porphyrins.

Its indispensability for lactic acid bacteria is at least partly based on being a precursor of serine, which is required in purine biosynthesis, and involvement in one-carbon metabolism via the glycine cleavage system. The fact of a functional folate biosynthesis pathway in LAB underscores glycine's importance. This study shows that the growth of these bacteria depends on glycine, rather than purines, and it is proposed that it may function as a key component of purine biosynthesis in addition to its role in other metabolic activities.

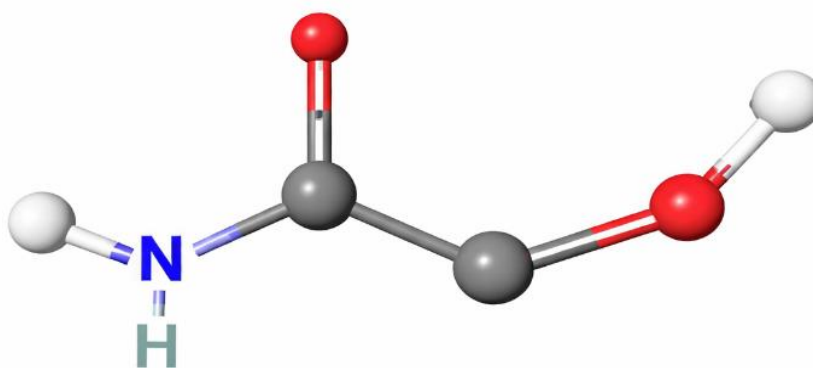


Figure 23. Glycine 2-aminoacetic acid retrieved from PUBCHEM CID 750

LAB Biotechnological Valorisation of Plants

Fermentation is commonly used in the food and beverage industry to make items such as beer, wine, and vinegar by fermenting plant sugars with yeasts and bacteria. In addition, fermentation elevates the nutritional profile of plant foods, as evidenced by soybeans becoming soy sauce, tempeh, and miso. In all 3 cases, the food tastes better and is easier to digest with increased availability of nutrients. Bioconversion operations are also used to produce high-value by-products, including enzymes, organic acids, and other metabolites that find use in the textile, paper, and food sectors (Alrefaey et al., 2021). The most apt example of the role of bioconversion for developing ingredients for BPs is associated with the fermentation of plant sugars to lactic acid. Lactic acid bacteria (LAB) are known to play a significant part in the bioconversion of secondary plant metabolites and act as biological catalysts for the transformation of the structure and function of plant constituents. Their enzymatic machineries, including glycosidases, esterases, decarboxylases, and reductases, can specifically and selectively cut the glycosidic bond or modification of the involved phenolic structure(s), leading potentially to a higher bioavailability (stability or biological activity) of physicochemicals (Naidu et al., 1999).

1.7. LAB Enzymes and Secondary Metabolite Activation/Inactivation

Lactic acid bacteria (LAB) harbor a various number of enzymes, such as β -glucosidases, esterases, phenolic acid decarboxylases, oxidoreductases, and proteolytic enzymes able to modify plant secondary metabolites through their cleavage or synthesis. Such enzymatic conversions are particularly important in the context of medicinal plant valorization, as many secondary metabolites occur as conjugates or polymers with low bioactivity. LAB may transform these compounds into more available or active forms through enzyme hydrolysis or redox reactions, thus increasing the nutraceutical or therapeutic value of herbal products. For example, LAB β -glucosidases may cleave the glycosidic bonds in flavonoid glycosides, which benefits absorption and bioactivity, as demonstrated by the conversion of daidzin to daidzein, resulting in an improved antioxidant capacity and estrogenicity. LAB enzymes are further involved in the detoxification of anti-nutritional or toxic physicochemicals like tannins, alkaloids, and saponins by hydrolyzing esterified polyphenols or degrading alkaloids (e.g., solanine), thus decreasing their toxicity while improving functional food safety/efficacy. The enzymatic behavior of LAB is strain-dependent and depends on the genetic, environmental, and fermentation conditions, and with the development

of genomics and metabolomics we have been provided with tools to select LAB strains for purposeful biotransformations. This allows for biotechnological valorization of medicinal plants such as those from areas like Western Algeria with distinct physicochemical profiles that may (or may not), however, require specific processing by microbial strains and ultimately supports sustainable bioprocessing in research and development.

Table 7. Important LAB enzymes and their functions

Enzyme Name	Target Substrate	Reaction Type	Functional Contribution	LAB Genera Examples
β -Glucosidase	Flavonoid glycosides	Hydrolysis (glycosidic bond cleavage)	Releases aglycones, enhancing bioactivity and bioavailability	<i>Lactobacillus</i> , <i>Leuconostoc</i> , <i>Pediococcus</i>
Esterase	Esterified phenolics & lipids	Hydrolysis (ester bond cleavage)	Reduces bitterness, releases aromatic compounds and improves digestibility	<i>Lactococcus</i> , <i>Lactobacillus</i>
Tannase	Hydrolyzable tannins	Hydrolysis (tannin ester bonds)	Reduces astringency and toxicity of tannins	<i>Lactobacillus</i> , <i>Enterococcus</i>
Phenolic Acid Decarboxylase	Hydroxycinnamic acids (e.g., ferulic acid)	Decarboxylation	Generates volatile phenols; improves antioxidant activity	<i>Lactobacillus</i> , <i>Weissella</i>
Protease	Plant proteins	Hydrolysis (peptide bond cleavage)	Increases digestibility,	<i>Lactobacillus</i> , <i>Streptococcus</i>

			releases bioactive peptides	
Glutamate Decarboxylase	Glutamate	Decarboxylation	Produces GABA, offering neuroprotective and antihypertensive effects	<i>Lactobacillus brevis</i> , <i>L. plantarum</i>
Laccase-like Enzyme	Phenolic compounds (e.g., catechols)	Oxidation (polyphenol oxidation)	Oxidizes polyphenols to quinones; may affect color and antimicrobial properties	<i>Lactobacillus spp.</i> (rare)
Nitrate Reductase	Nitrate ions	Reduction	Detoxifies nitrates and contributes to anaerobic energy metabolism	<i>Lactobacillus</i> , <i>Carnobacterium</i>

Source: according to the works of (Lara-Espinoza et al., 2021), Natalia et al., 2020; and Niazian, 2019).

1.8. Lactic Acid Bacteria Fermentation

Techniques such as fermentation, enzymatic breakdown, and the use of Lactic Acid Bacteria (LAB) have proven to be particularly effective in improving the potency and stability of plant-based compounds. For instance, LAB fermentation is used to enhance the antimicrobial properties of plants like *Salvia officinalis* (sage), which has been traditionally used for its healing properties. Through fermentation, LAB convert complex plant compounds into simpler, bioactive forms, increasing the therapeutic potential of the plants and making them more effective for use in pharmaceuticals, nutraceuticals, and cosmetics. This optimization of plant bioactive compounds helps ensure that these plants can be utilized in more targeted and effective treatments, offering significant benefits to both medical and commercial sectors (El Sheikha, 2017; Niazian, 2019).

Table 8. Relevant works reporting beetroot fermentation utilizing lactic acid bacteria

Fermentation Agents	Conditions	Key Findings	References
Lactic acid bacteria	pH 6.5 at 37°C	Increase in free phenolic acids. Decrease in conjugated phenolic acids. Highlights beetroot's potential as a non-dairy probiotic.	(Natalia et al., 2020)
<i>L. Plantarum</i> <i>L. paracasei</i>	pH 6.5 at 30°C	<i>L. Plantarum</i> and <i>L. paracasei</i> showed significant growth in beet juice fermentation. -Suggests beetroot's suitability for producing probiotic beet juices.	(Jafar et al., 2019a)
<i>L. brevis 0944</i> <i>L. paracasei</i>	NM	Fermented beetroot juice had a positive impact on gut microflora and metabolic activity in rats.	(Klewicka et al., 2015)
<i>L. acidophilus</i> <i>L. casei</i> <i>L. delbrueckii</i> <i>L. plantarum</i>	At 30°C for 48 h	<i>L. acidophilus</i> and <i>L. plantarum</i> produced more lactic acid, reducing pH to below 4.5. Viable cell counts remained at 10 ⁶ –10 ⁸ CFU/ml after 4 weeks of cold storage.	(Kyung et al., 2005)
Lactic Bacteria	,	Lactic fermentation led to the conversion of sugars into lactic acid.	(Foss et al., 2023)
Co-culture <i>L. acidophilus</i> and <i>L. plantarum</i>	Anaerobic fermented for 24 and 48 h	Antibacterial activity against <i>Listeria monocytogenes</i> .	(Firoozirad et al., 2021)

		48 h fermented juice had cytotoxic activity against human liver cancer cells.	
<i>Lactic fermentation using LAB</i>	Fermentation spray drying	Fermentation produced LAB-rich beetroot juice.	(Janiszewska-Turak et al., 2022)
<i>L. plantarum</i> <i>L. paracasei</i>	Fermentation	Lactic fermentation altered the properties of beetroot and carrot juices.	(Janiszewska-Turak et al., 2023)

NM: not mentioned

Part 2

Experimental research

Materials and Methods

1. Statement of objective

Traditional laboratory practices rely on specialized media formulations, such as the widely used MRS (de Man, Rogosa, and Sharpe) medium, to cultivate LAB. These media come at a significant financial expense, can be subject to ruptures, posing a challenge for resource-constrained laboratories and industries. The main target of this study is to research and develop a new growth medium made from plant sources for the Lactic acid bacteria. Working on Algerian plants (beetroot, carob, and soybean), the research intends to develop a medium that can be contrasted with MRS with comparable and measurable metrics.

1.1. Hypothesis

Our hypothesis is the following:

- H1: Algerian plants, individually or combined, can replace traditional growth media.
- H2: Beetroot, carob, and soybeans' nutritional can satisfy minimal trophic requirement of the lactic acid bacteria chosen.
- H3: Potential additive synergies could enhance plant-based growth medium.

The resulting medium will effectively support LAB genera growth

2. Sampling

2.1. Plant

Beet pulps, Carob, and soybeans were obtained from local farms in Mascara, Algeria (GPS: 35.401864, 0.139919) in the spring season during the period from March to April. The identification of the vegetal specimen was conducted by Mr. Zahafi, affiliated with Department of agronomy university of Mustapha stambouli - Mascara, in accordance with the established criteria for plant identification, ensuring the accurate classification and validation of the specimen. They were then thoroughly cleaned to remove soil and debris. The leafy parts were carefully trimmed using a sterile scalpel, retaining only the necessary sections. The materials were then peeled, ground, and air-dried at room temperature (around 25°C) in a well-ventilated area. This natural dehydration process involves moisture removal through air circulation at ambient temperatures, without the use of artificial heat. After drying, the materials were ground into a fine powder using

a mechanical grinder. They were then stored in airtight containers in a cool, dry place to preserve their stability.



Figure 25.plants part used; Beetroot, Carob, Soybean

2.2. Lactic Acid Bacterial Strains

For all the following work, the three stated lactic acid bacteria were utilized: namely, *Lactobacillus fermentum* (NBRC15885), *Enterococcus durans* (JCM8725) and *Lactobacillus plantarum* (JCM1149) were used for this research (Benamara et al., 2016). These strains were identified through RNA 16S sequencing by *LGC Genomics* (Berlin, Germany), with sequence analysis

performed using the NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST/>). When cultivating, the strains were subject to two consecutive propagations in MRS broth at 37°C for 24 hours.

2.3. Validation of Criteria:

- **Availability:** All three strains are widely distributed and commonly found in natural and industrial fermentation processes, providing high reliability for the study's reproducibility.
- **Fermentation Profile:** The selection of strains with different fermentation profiles ensures that the medium is evaluated from multiple angles, not just for basic sugar fermentation, but also for more complex substrates like oligosaccharides and fibers.
- **Stress Tolerance:** The inclusion of *Enterococcus durans*, known for its resilience to adverse conditions, brings a pragmatic assessment to the study.

2.4. Purity verification

Verification of the Lactic Acid Bacteria (LAB) purity was ensured using several key microbiological tests. Gram staining: confirmed the Gram-positive nature of the bacteria. The oxidase enzyme test: LAB strains are oxidase-negative; Absence of bubble formation. The catalase enzyme test: LAB strains are catalase-negative; absence of color change.

First Stage: Beetroot with different chemical add-ins for enhanced performance.

2.5. Physicochemical analyses

The study involved grinding 100 g of dried beetroot powder to a consistent particle size, followed by established laboratory techniques to measure its nutrient profile. reference standards for overall sugar content, protein, moisture content, fat percentage, fiber content, and moisture level were prepared in accordance with established guidelines. Afterward they were then analyzed using spectrophotometry, Soxhlet extraction for fat content, and weight-based methods for moisture and fiber measurement. The phenol-sulfuric acid protocol was used for total sugar analysis, and protein quantification was measured via the Kjeldahl method. To ensure reliability and consistency, all analysis was realized in triplicate (Ingle et al., 2017; Otalora et al., 2020; Shi et al., 2021).

2.6. Titratable Acidity

The titratable acidity was determined by titrating an acidic mixture with a base solution of available concentration, following the guidelines of Sujka *et al.* This measurement indicates the quantity of acid in the sample, presented in concentration values. To perform the procedure, NaOH solution is added to 20 mL of juice containing an acid/base indicator, and the color shift is notable until it stabilizes. The titratable acidity is then calculated by assessing the volume of NaOH consumed, considering its concentration, and multiplying the result to find the titratable acidity in 100 mL of juice (Sujka et al., 2018). juice.

2.7. Total Sugar Assessment

For quantifying the overall sugars in beet juice, the phenol-sulphuric acid method was employed. A sugar assay reagent solution was prepared abiding by the manufacturer's guidelines. Next, 10 mL of beet juice was transferred into a 50 mL beaker, diluted in distilled water to achieve a 50 mL volume. A 1 M sodium hydroxide (NaOH) solution was prepared using a graduated burette. The beetroot juice blend was then subject to heating in a water bath until it boiled. The reagent was added drop by drop to the blend while stirring. The resulting color compound was measured to determine the total sugar content.

2.8. Inoculum Preparation

The frozen strain is quickly thawed by submerging it in a 37°C water bath then inoculated into the MRS broth using a sterile inoculation loop. The tubes containing the inoculated strains are incubated at (35-37°C for *lactobacilli*, 37-40°C for *enterococci*) from 24 up to 48 hours. Once favorable Bacterial growth is observed after incubation, the bacteria are transferred to a gelatinous MRS culture medium propagate properly.

2.9. Beetroot-formulated media fermentation

In our research aimed at developing a beetroot-based medium as a viable alternative to the MRS medium for cultivating lactic acid bacteria, the study was structured in several experimental phases. Initially, the effects of beetroot juice alone were examined. Subsequently for 500 ml of the Beetroot juice we auditioned these fixed additives, including meat extract (5 g), yeast extract (5 g), peptone (10 g), and threonine (0.5 g), were introduced to explore their synergistic effects, using the same concentrations as in the standard MRS medium for 500 g of medium. The study then progressed to incorporate various chemical agents, following the MRS medium composition:

Magnesium sulphate (0.1 g), Manganese sulphate (0.05 g), Sodium acetate (2 g), Ammonium citrate (2 g), di-potassium phosphate (2 g), and zinc oxide (0.05 g). These components were integrated into the beetroot and additive mix, allowing for the examination of their interactions. This multi-phase approach provided valuable insights into the potential of beetroot-based media as a replacement for conventional MRS medium in the cultivation of lactic acid bacteria.

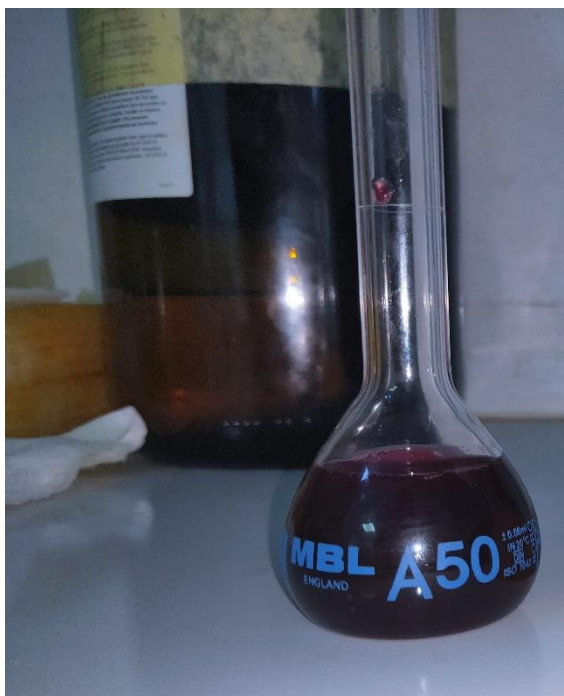


Figure 26. aspect of one of the formulation used

2.9.1. Colorimetric analysis of the fermentation medium

The color evaluation involved the utilization of the spectrophotometer to conduct colorimetric measurements to assess the growth dynamics of three lactic acid bacteria strains. Samples were extracted from the initial solution at 0, 12, and 24 hours. The colorimeters' analysis of the BJFA-MS inoculated with 10^6 UFC/ML, providing a quantitative assessment of growth based on changes in color intensity over the specified time intervals (Śliżewska & Chlebicz-Wójcik, 2020). This parameter aim to evaluate and expose an understanding of the fermentation and growth dynamics through another visual aspect.

2.9.2. Selection of BJFA-MS and Fermentation Comparative Analysis

The contrasting evaluation of fermentation between the BJFA-MS and MRS mediums involved fermenting chosen lactic acid bacteria strains in both and monitor pH, acidity, and OD. metrics assessed were the maximal growth specific speed (μ_{max}), generation time (G), acid production speed (Q_{ac}), and productivity in (g/L.h), representing the quantity of acid produced per hour. Throughout the Fermentation experiments samples collected at regular intervals for analysis. The collected data were then subjected to statistical analysis.

2.10. Testing growth on agar plates

A 100 g of dried vegetable powder was solubilized in 250 ml, to which 250 ml of distilled water containing pre-solubilized additives and 4.6 g of agar powder was added, then homogenized and sterilized for 120 degree for 3-5 minute, with the add ins incorporated with namely: Meat and Yeast extract, Peptone and Tween 80. Once the medium is homogenous it's poured Petri plates. Inoculation of 0.1 ml volume from tested bacteria solutions with a turbidity level of 1.5×10^8 CFU/ml (corresponding to 0.5 McFarland). We incubate at 37°C for 24 hours. Documenting growth, macroscopic aspect (Andualem & Gessesse, 2013).



Figure 27. Growth medium of Agar for the formulations

2.11. Statistical analysis and fermentation parameters calculation

All experiments were performed in triplicate. The results are expressed as the mean \pm SD (standard deviation). Statistical analysis of the experimental data was conducted using the SPSS 13.0 software. Calculation of fermentation parameters involves determining the kinetic culture parameters. The specific microbial growth rate (μ) can be calculated through the utilization of the semi-logarithmic method to plot $\ln (X/X_0)$ against time, as for the time of generation it's expressed as

$$G = \ln 2 / \mu_{\max} .$$

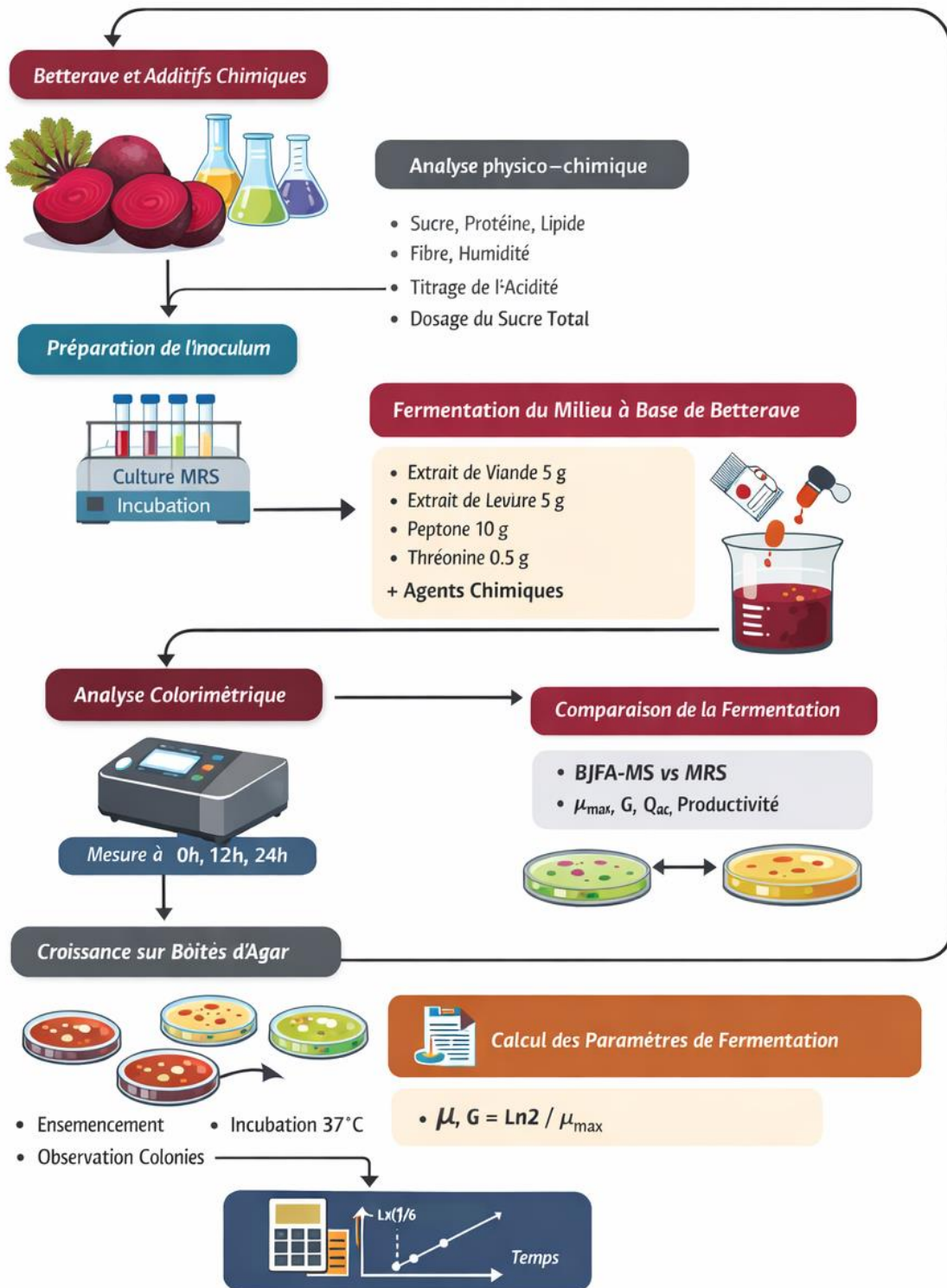


Figure 28. logigramme des successions des analyse of first stage

Second Stage: Beetroot with A peptone substitute (Carob pods)

2.1. Physico-chemical Analyses

Physico-chemical analyses were conducted on 100 g samples of dried, finely ground beetroot and soybean powders using standard chemical assays. Nutritional components, including total sugars, protein, water, fat, fiber content, and humidity, were quantified following standard protocols. Calibration standards were prepared for accurate measurement, and chemical reagents were handled according to standard procedures. Data accuracy was ensured through statistical analysis and validated by cross-referencing with independent laboratory results (Janiszewska-Turak et al., 2023). Acidity and total sugar quantity in beet and soybean juices were determined using standard methods. Acidity was measured by titration with a standardized NaOH solution to a pH endpoint of 8.2, following ISO 750:1998. Overall sugar content was quantified using the phenol-sulfuric acid method, involving a reaction with phenol and sulfuric acid, followed by spectrophotometric measurement at 490 nm. Both methods followed the protocols of (DuBois et al., 1956) for the phenol-sulfuric acid method and ISO 750:1998 for titratable acidity (International Organization for Standardization, 1998).

2.2. Inoculum Preparation

Preserved bacterial strains were reanimated by transferring them to culture media tailored to each strain. For the case of Lactic acid bacteria, the reanimation medium was MRS broth. The frozen strains were thawed and inoculated into the respective media, followed by incubation at recommended temperatures to promote bacterial growth.

2.3. Vegetal Formulated Media Fermentation

A systematic series of experiments was conducted to evaluate the potential of carob and beetroot-derived media for cultivating Lactic Acid Bacteria (LAB). The standalone effects of each ingredient were initially examined, following standard protocols for media formulation and LAB cultivation, as outlined by earlier studies (Gänzle et al., 2000; Gobbetti et al., 2010). After assessing the individual effects, fixed additives, including meat extract (5 g), yeast extract (5 g), and selective chemical agents like sodium acetate (2 g) and ammonium citrate (2 g), were incorporated into the media to test their impact on LAB growth and metabolic activity, using the same concentrations as in the standard MRS medium for 500 g of medium (Prete et al., 2021). Subsequently, we investigated possible combinations of the two plant sources (soybean and beetroot), both

individually and in combination, with supplementation, to compare their results with those of the standalone media. This approach aimed to assess potential complementary effects from the respective plants, based on the differences in their nutrient composition and attributes, as described in previous research on plant-based media for microbial cultivation (Yang et al., 2024).



Figure 29. Aspect of the formulation based of Beetroot and Carob

2.3.1. Fermentation kinetics and analysis

This contrasting assessment included fermenting the studied lactic acid bacteria strains in both MRS medium and other formulated media derived from various tested plants. Throughout the fermentation process, key parameters such as acidity, pH, and OD were meticulously monitored. The metrics evaluated included μ_{max} (maximum specific growth rate), G (generation time), Q_{ac} (acid production rate), and productivity (g/L.h). Throughout the fermentation, samples were collected at regular intervals and analysed. The data obtained were then subjected to statistical analysis to elucidate the differences and similarities between the various media (Garrote Achou et al., 2025; S. Yang et al., 2024).

2.4. Testing Growth on Agar Plates

A total of 100 g of dried vegetable powder for each plant was solubilized in 250 ml, to which 250 ml of distilled water containing pre-solubilized additives and 4.6 g of agar powder was added, then homogenized and sterilized for 120 degree for 3-5 minute. Inoculation with standardized turbidity levels was performed, followed by incubation to observe growth dynamics, macroscopic appearance, and colony characteristics.

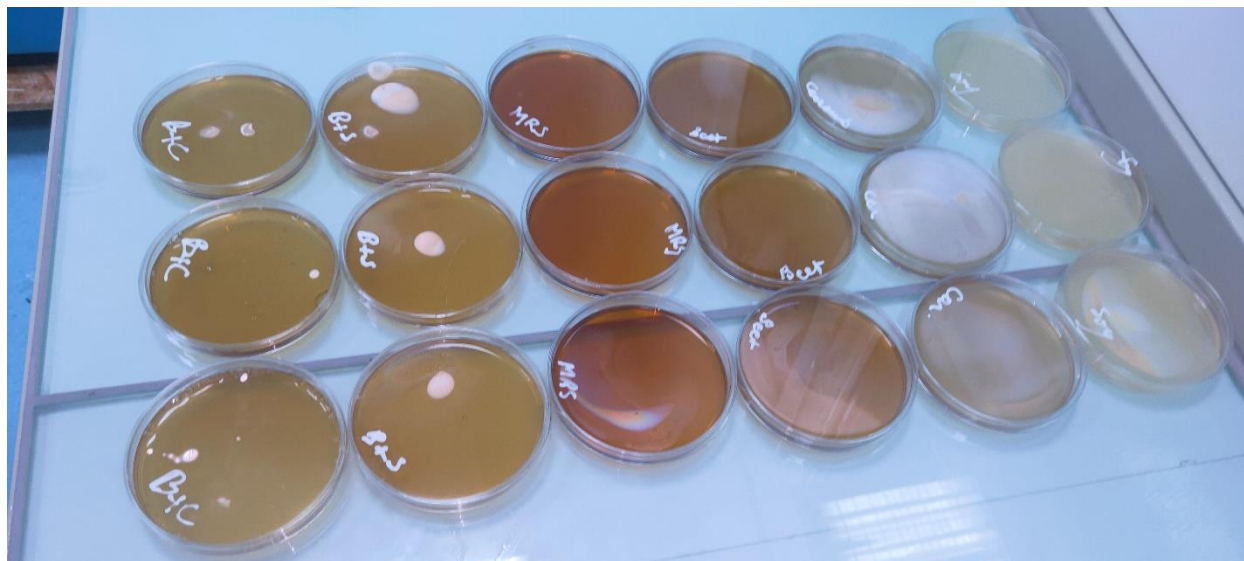


Figure 30. aspect of growth of agar for the medium formulated

2.5. Statistical Analysis and Fermentation Parameters Calculation

To assess the performance of various media types on bacterial growth and productivity, a detailed analytical framework was utilized. Fermentation experiments were performed in triplicate with duplicate analyses per sample to ensure data robustness. Statistical evaluations were conducted to calculate key fermentation parameters, including specific microbial growth rates. A one-way ANOVA was employed to detect significant differences in parameters such as maximum specific growth rate (μ_{max}), generation time, acid production rate (Q_{ac}), and productivity across the media.

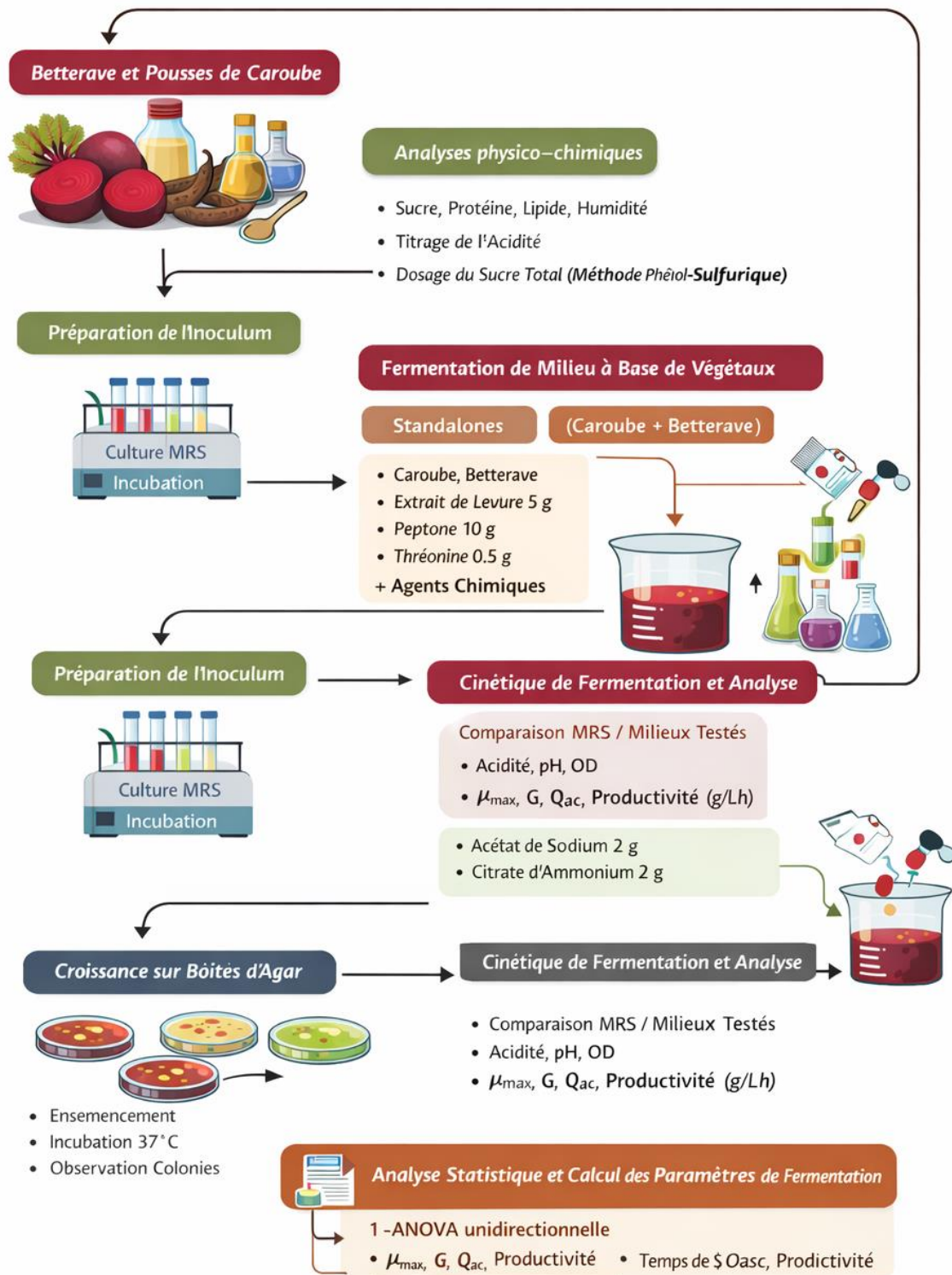


Figure 31. logigramme des succession des analyse of second stage

2.6. Physico-chemical Analyses

Physico-chemical analyses were conducted on 100 g samples of dried and finely ground beetroot and soybean powders using standard chemical assays. Nutritional components, including total sugars, protein, water, fat, fiber content, and humidity, were quantified following standard protocols. Calibration standards were prepared for accurate measurement, and chemical reagents were handled according to standard procedures. (Janiszewska-Turak et al., 2023) Titratable acidity and total sugar content in beet and soybean juices were determined using a pH meter (Model Metrohm pH-Meter 632 AG CH-9101 Herisau -Swiss made). Titratable acidity was measured by titration with a standardized 0.1 N NaOH solution of 98% purity to a final pH of 8.2, according to ISO 750:1998. Total sugar content was quantified using the phenol-sulfuric acid method; a phenol solution was prepared at a 5% (w/v) concentration (5 g of phenol per 100 mL of water) with a purity of 99%, involving a reaction with phenol and sulfuric acid, followed by spectrophotometric measurement (Model SPECORD® 50 PLUS BU-TTF from Analytik Jena company - Germany) at 490 nm. Both methods followed the protocols of DuBois et al. (DuBois et al., 1956) regarding the phenol-sulfuric acid method and ISO 12846:2012 for titratable acidity. All measurements were performed in triplicate.

2.7. High-Performance Liquid Chromatography Analysis

The soybean and beetroot medium contents were determined using HPLC analysis, with compounds identified according to Fluka and Sigma Aldrich standards, with a purity of 99.5%, using Shimadzu equipment (USA Manufacturing Inc, USA) consisting of two LC-20AD pumps, a CBM-20A controller, a SIL-20AC column oven, and an SPD-20AV UV/Vis spectrometer. Accurately weighed amounts of soybean and beetroot powder samples were placed in plastic test tubes. Then, 1 mL of methanol containing 1% ascorbic acid was added. The components were mixed well using a vortex device, and then incubated in an ultrasonic bath for 20 min at 20°C. After incubation, the samples were centrifuged at $3,920 \times g$. From each test tube, 1 mL of the extract was individually collected and recentrifuged at $22,579 \times g$. Subsequently, 500 μL of each extract was transferred into HPLC vials for analysis. The concentration and retention time of each compound were expressed in ppm, following the protocol described by Kelebek et al.¹⁰

2.8. Vegetal-formulated media Fermentation

A systematic series of experiments was conducted to evaluate the potential of soybean and beetroot media mixtures for lactic acid bacteria (LAB) cultivation. Initially, the individual effects of each component were assessed using standard protocols for media formulation and LAB cultivation, as described by Gänzle et al. and Gobbetti et al. (Gänzle et al., 2000; Gobbetti et al., 2010). Subsequently, selected additives, including meat extract (5 g), yeast extract (5 g), and chemical agents such as sodium acetate (2 g) and ammonium citrate (2 g), at concentrations ranging from 1 g/L to 0.1 M, were incorporated into the media to evaluate their influence on LAB growth and metabolic activity, using the same concentrations as in the standard MRS medium for 500 g of medium (Prete et al., 2021). Next, we investigated the possible combinations of the two plant sources (soybean and beetroot), both individually and in combination, with supplementation, to compare their results with those of the standalone media. This approach aimed to assess potential complementary interactions between the two plant sources, considering their distinct nutrient profiles and functional attributes, as highlighted by previous studies on plant-based media for microbial cultivation (Yang et al., 2024)

2.8.1. Fermentation kinetics and analysis

A comparative study was conducted by fermenting selected lactic acid bacterial strains in MRS medium and formulated media derived from various tested plant sources. The fermentation experiments were performed under strictly controlled conditions, including temperature (37°C) and agitation (150 rpm). (S. Yang et al., 2024) Key parameters, such as acidity, pH, and optical density (OD), were monitored throughout the fermentation process. The fermentation kinetics were analyzed by assessing parameters such as the maximum specific growth rate (μ_{max}), generation time (G), acid production rate (Q_{ac}) and productivity (g/L/h), which represents the amount of acid produced per hour (Yeboah et al., 2023) Samples were collected at regular intervals for comprehensive analysis, including pH measurements, optical density, and acid concentration determination. (Abedin et al., 2024) These methods were adapted from established protocols for LAB fermentation. As well, measurements were performed in triplicate, ensuring consistency and reproducibility of the results. (Garrote Achou et al., 2025)

2.9. Testing Growth on Agar Plates

Agar plates were prepared using fermentation-derived media a total of 100 g of dried vegetable powder -for each plant- was solubilized in 250 ml, to which 250 ml of distilled water containing pre-solubilized additives and 4.6 g of agar powder was added, then homogenized and sterilized for 120 degree for 3-5 minute. For inoculation, bacterial strains were adjusted to a standardized optical density (OD) of 0.1 at 600 nm (approximately 10^8 CFU/mL), according to standard practice for ensuring consistent bacterial concentration. (Hujanen et al., 2001) Following inoculation, the plates were incubated at 37°C for 24 to 48 hours under aerobic conditions to evaluate growth dynamics, macroscopic appearance, and colony characteristics, including size, shape, and color. Incubation was conducted at 37°C with a maintained relative humidity of 90%. (Śliżewska & Chlebicz-Wójcik, 2020) Colony characteristics such as morphology (e.g., round, smooth, or rough), color (e.g., white, creamy), and texture were recorded to assess the growth performance of the strains across different media.

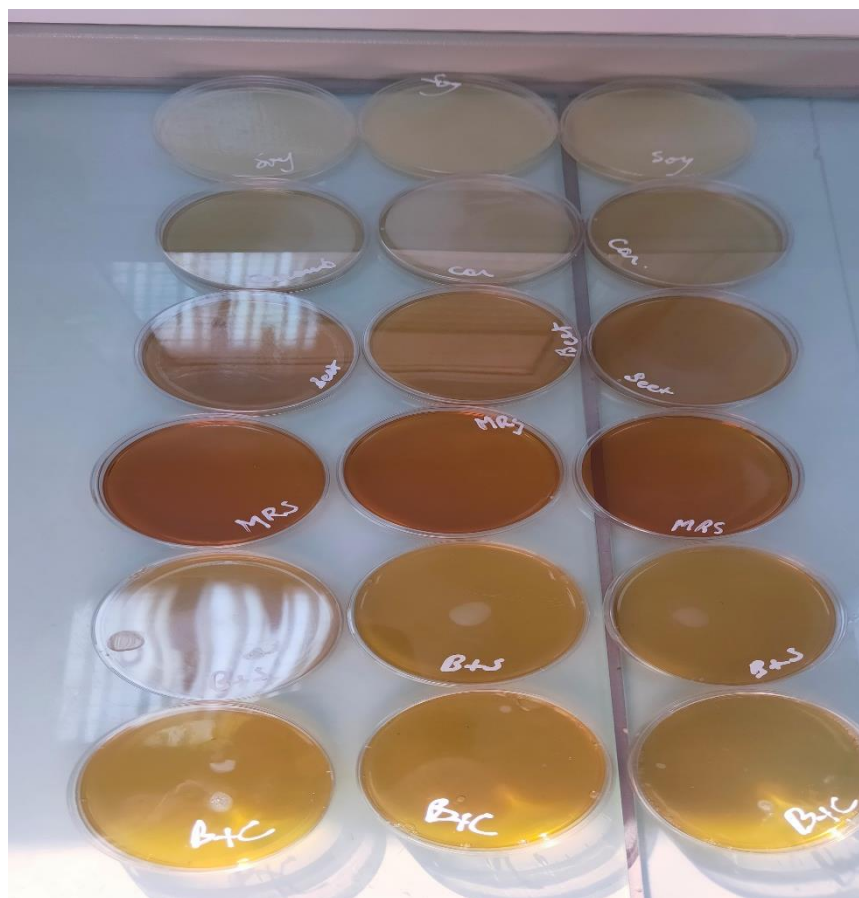


Figure 32. Petris plates of the formulated medium inoculated with the tested bacteria

2.10. Statistical Analysis and Fermentation Parameters Calculation

To assess the effectiveness of the formulated medium on bacterial growth, a detailed analytical framework was implemented. Fermentation experiments were performed in triplicate. Statistical analyses were carried out to evaluate key fermentation parameters, including specific microbial growth rates. A one-way variance analysis (ANOVA) was employed to detect significant differences in parameters such as maximum specific growth rate (μ_{max}), generation time, acid production rate (Q_{ac}) and productivity across media, thereby highlighting statistically significant variations.

Selectivity test

Four media, MRS (control), BJFAMS, CBB, and SBB were prepared ($pH\ 6.6 \pm 0.1$) and tested for selectivity against *Staphylococcus Aureus ATCC 43300* and *Escherichia coli ATCC 25922* with the agar plating methods for a period of 72 H. Each strain, standardized to 10^7 CFU/mL, was inoculated (10 μ L) on triplicate plates and incubated at 37 °C for 24–48 h. Colony counts, morphology, and growth intensity were recorded.

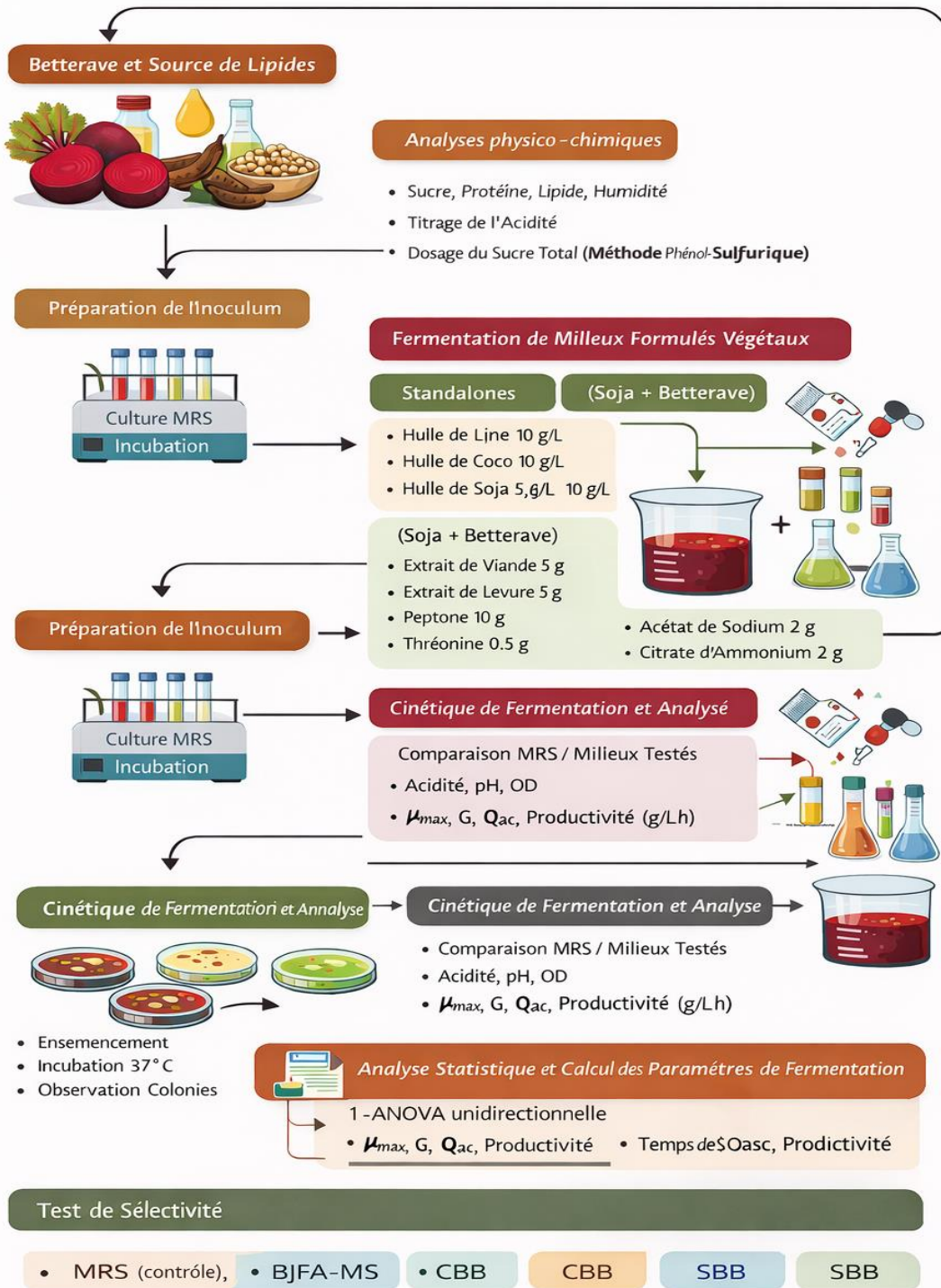


Figure 33. logigramme of the third stage

Results and discussion

First Stage Results

2.1. Purity test results

Table 9. purity test verification

	<i>L. plantarum</i>	<i>E. durans</i>	<i>L. fermentum</i>
Gram	+	+	+
Oxidase	-	-	-
Catalase	-	-	-
Sporulation	Non sporulated	Non sporulated	Non sporulated

2.2. Physico-chemical results

In the results section, we present findings from the physico-chemical characterization of the Beetroot powder

Table 10. Photochemical analysis results of beetroot powder.

Parameter	beetroot powder
pH	5.5
Acidity titrble m. eq	13.02
Total sugar	67
le plus abundant	Sucrose
water content g	87.6
Protein	31.09
FAT%	1.32
cellulose(fiber)	18.17
humidity	8.45
Optical density	2.16

Our findings from table 10 indicate a pH value of 5.5, coherent to Jafar et al., 2004 study (Jafar et al., 2019b) where pH values encompassed between 5.5-6.5. pH evaluation is a basic stepping stone to evaluate suitability of the environment (Rakin et al., 2004; Xu et al., 2020). While Titratable acidity function as a marker of organic acid impact in foods. We observed 13% titratable acidity in beet juice hinting presence of organic acids and this finding aligns with Chen Chen et al., 2017, Lee et al., 2019 and Johnson et al., 2022 findings that encompassed it in a range of 10 to 15 mEq in similar juice. the optimal sugar concentration range (50-70 g/L) known to support lactic acid bacteria growth and lactic acid production and the Overall sugar content in the beet-based medium, measured at 67 g/L concluding to suitable sugar availability. This data is consistent with findings by Bujna et al., 2017 and Venegas et al., 2019, reporting a 60 to 70 g/L sugar concentrations from same origin. The work of Kazimierzak et al., 2014 on dry matter content in beetroot juices revealed notably lower values compared ours. Moreover, the extract and density values presented in our study exceed those in prior works Janiszewska-Turak et al., 2022, likely attributable to variations in vegetable maturity and experimental timing.

2.3. Fermentation

The confirmed suitability of formulated medium, the fermentation of beetroot juice alone and in different formulations by 3 lactic acid bacteria namely: *E. durans*, *L. fermentum* and *L. plantarum*. Initial inoculum was calibrated to 10^7 log CFU/mL Figure 23, with OD serving as an indicator of bacterial growth and medium consumption. Multiple contrasts were observed between the beetroot juice alone and the remaining formulation juice. Note that the medium: Beetroot Juice with Fixed Additives -peptone, meat and yeast extract, tween80 will be referred to as 'BJFA' from here forth.

As a start, beetroot juice alone revealed less growth for all tested lactic strains throughout the fermentation period, this can be related to the lack of nutritional properties of beetroot alone and that it's not a stand-alone media for lactic acid bacteria which are known to be exigent in their trophic profiles. The three bacterial strains exhibited significantly enhanced growth when the initial medium was supplemented with: meat and yeast extract, peptone, and Tween 80. Notably, the growth recorded during fermentation with these additives was considerably higher compared to the fermentation of beetroot juice alone. Remarkably, the inclusion of: magnesium sulfate, ammonium citrate, and di-potassium phosphate in the beetroot-based medium led to a marked improvement in bacterial growth. However, when compared to the MRS medium, bacterial proliferation remained less pronounced. A promising formulation that showed success in this experiment was the combination of beetroot juice with magnesium sulfate (BJFA-MS), achieving results comparable to the MRS medium and following a similar growth pattern.

Furthermore, the findings of this study align with those reported by Muneeb et al. (2019), who observed significant growth in *Lactobacillus plantarum*, with a gradual increase in viable cell count over time when using beetroot juice (Malik et al., 2019b). A similar approach was employed in the study by Wang et al. (2015), whose method involved processing beetroot juice with *Lactobacillus plantarum* to produce fermented beet extract. Their results emphasized that beetroot

provides the essential nutrients required for the growth and development of *Lactobacillus plantarum*, facilitating lactic acid production and successful fermentation of the beetroot medium (Wang et al., 2015).

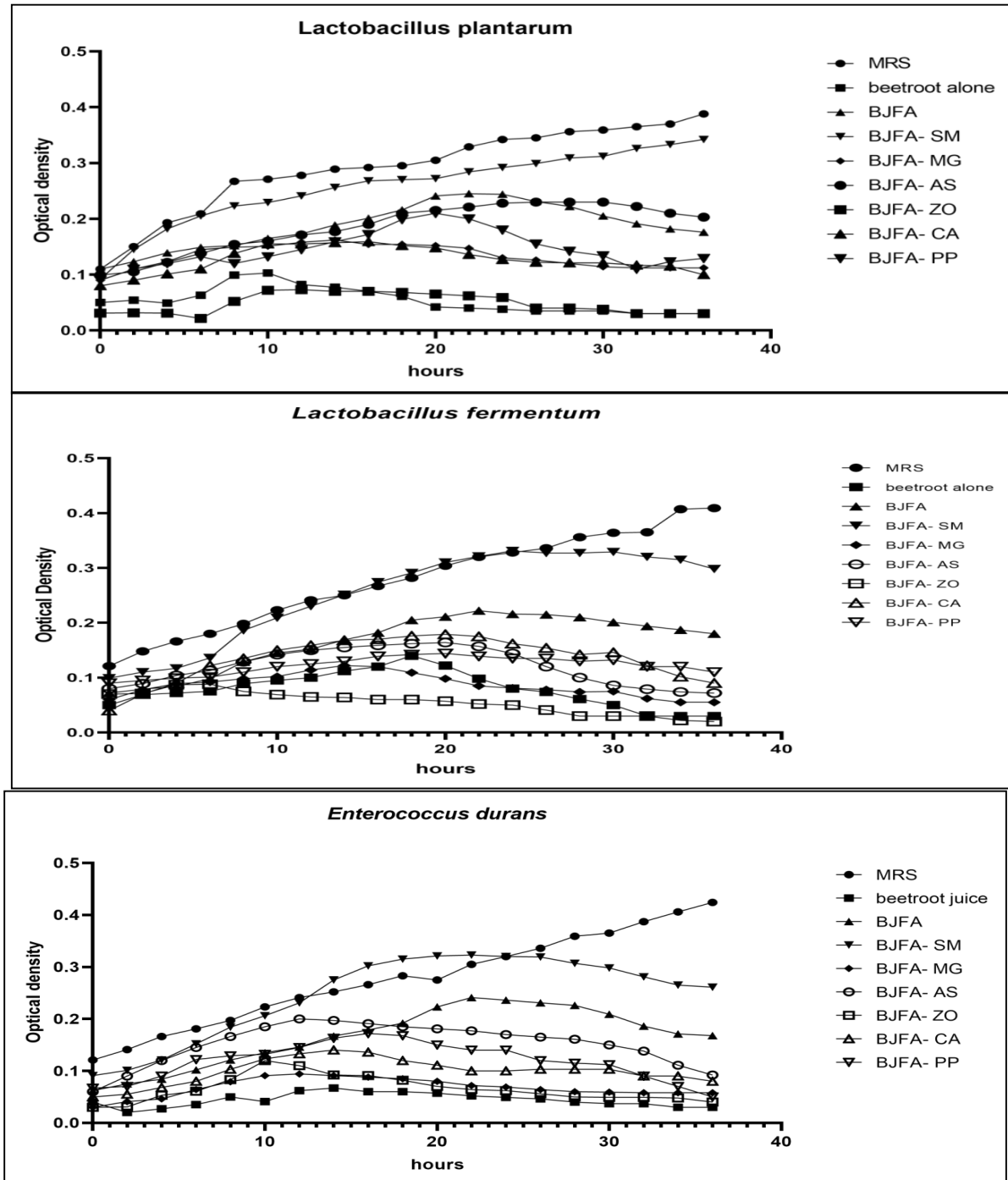


Figure 13. Fermentation on different formulation from BJFA with *L. plantarum*, *L. fermentum* and *E. durans*; MRS: Man de Rogosa Sharp, SM: sulphate manganese, MG: sulphate Magnesium, AS: acetate sodium, ZO: zinc oxide, CA: citrate ammonium, PP: phosphate di potassium

2.4. Kinetic parameter of fermentation

Traditionally, Man de Rogosa Sharp (MRS) medium, is the standard for cultivating and propagating lactic acid bacteria so in order to prove that an alternative is considerable it has to compare to MRS in calculable metrics. The calculation of specific growth rate (μ_{\max} , h^{-1}) and generation time for each medium, as shown in Table 11, was performed based on data obtained from the fermentation process. While several formulations from the beetroot-based medium (BJFA) demonstrated higher specific growth rates, it is important to note that growth speed should be interpreted in conjunction with generation time for proper comparison with the reference medium.

Table 11. Results of kinetics fermentation of different BJFA with the lactic acid bacteria

Fermentations Media	μ_{\max} (h^{-1})			Generation time G (h)		
	<i>L.</i> <i>plantarum</i>	<i>E.</i> <i>durans</i>	<i>L.</i> <i>fermentum</i>	<i>L.</i> <i>plantarum</i>	<i>E.</i> <i>durans</i>	<i>L.</i> <i>fermentum</i>
MRS ¹	0.0138	0.0276	0.0242	50.22	25.11	28.64
Beetroot alone	0.030	0.091	0.044	23.10	07.61	15.75
BJFA ²	0.035	0.060	0.039	19.80	11.55	17.77
BJFA-MS ³	0.015	0.073	0.036	46.21	9.49	19.25
BJFA-MG ⁴	0.014	0.112	0.031	49.51	6.19	22.36
BJFA-AS ⁵	0.032	0.077	0.049	21.66	9.00	14.14
BJFA-ZO ⁶	0.010	0.109	0.009	69.31	6.36	77.01
BJFA-CA ⁷	0.043	0.081	0.022	16.12	8.56	31.50
BJFA-PP ⁸	0.032	0.031	0.025	21.66	22.36	27.72

1: MRS: Man de Rogosa Sharp, 2: Beetroot Juice Fixed Additives; 3: SM: sulphate manganese, 4: sulphate Magnesium Sulfate, 5: Acetate Sodium, 6: Zinc oxide, 7: Citrate Ammonium, 8: phosphate dipotassium

2.5. Color-metrics

The color of the juice can be precisely quantified using the Y^* , R^* , and G^* color values which represent the Yellow, Red/Green proportions, respectively (refer to Tables 12). Notably, beetroot juice exhibits the highest red value at the start of fermentation, with an R^* value ranging from 1.8 to 2.0, while the yellow value is initially absent (null). The Green value at the beginning is minimal, ranging from approximately 0.01 to 0.03. Upon fermentation with the lactic acid bacterial strain, significant changes in the color profile of the juice were observed. The Yellow value increased considerably, reaching between 25 and 30, while the green value (chlorophyll) rose to

approximately 0.37–0.40. A notable shift was the disappearance of the Red* value, which is typically attributed to the presence of betalain pigments in beetroot, with the Red* value dropping below 0. This alteration in the color indices is probably linked to the breakdown of nutritional components in the medium, driven by the increased lactic acid production and the corresponding reduction in pH.

Literature explains that pigments like carotenoids and betalains are sensitive to low pH levels. However, betalains tend to exhibit greater stability under acidic conditions compared to carotenoids. (Jackson, 2020; Marszałek et al., 2021; Sajjad et al., 2020) This could be due to the hydrolyzation process where glucose is obtained from betanin, which is a glycoside, and glucose and betanidin are created and since these LAB strains can use this glucose and betanidin as a sugar source (Marzo et al., 2021).

Table 12. colorimetric of the medium BJFA -MS

<i>Lactobacillus plantarum</i>			
Color	0h	12h	24h
Yellow	<0	13	28
Red	1.8	0.8	<0.0
Chlorophyll	0.01	0.19	0.40
<i>Enterococcus durans</i>			
Color	0h	12h	24h
Yellow	<0	10	25
Red	1.9	0.9	<0.0
Chlorophyll	0.01	0.20	0.37
<i>Lactobacillus fermentum</i>			
Color	0h	12h	24h
Yellow	<0	12	30
Red	2.0	1.1	<0.0
Chlorophyll	0.03	0.23	0.38

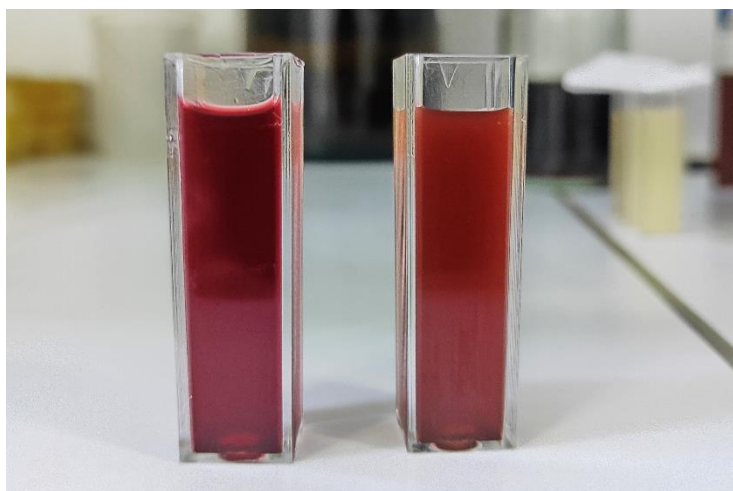


Figure 36. Visual aspect of the colorimetric results

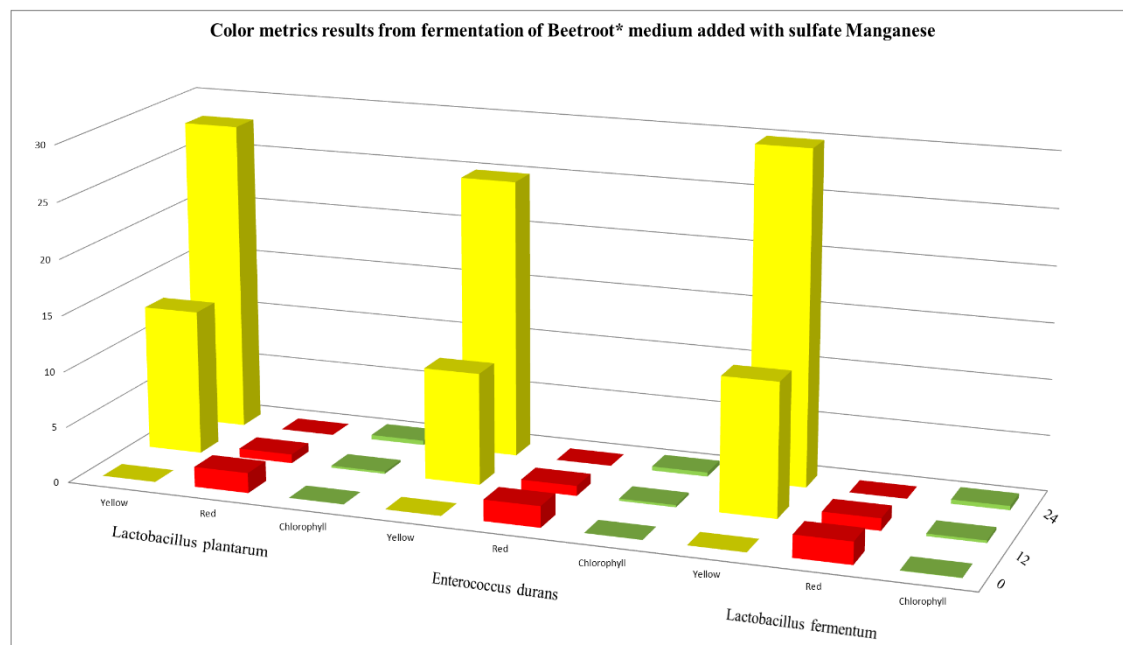


Figure 35. graphic representation of the colorimetric results for medium BJFA –MS for *L.plantarum*, *L. fermentum* and *E. durans*

2.6. Comparative Fermentation in BJFA-MS and MRS

The results presented here confer a contrasting representation of fermentation outcomes between BJFA-MS and MRS mediums for three selected lactic acid bacteria strains. These findings provide valuable insights into the suitability of BJFA-MS in fostering lactic acid bacteria fermentation and its implications for industrial applications.

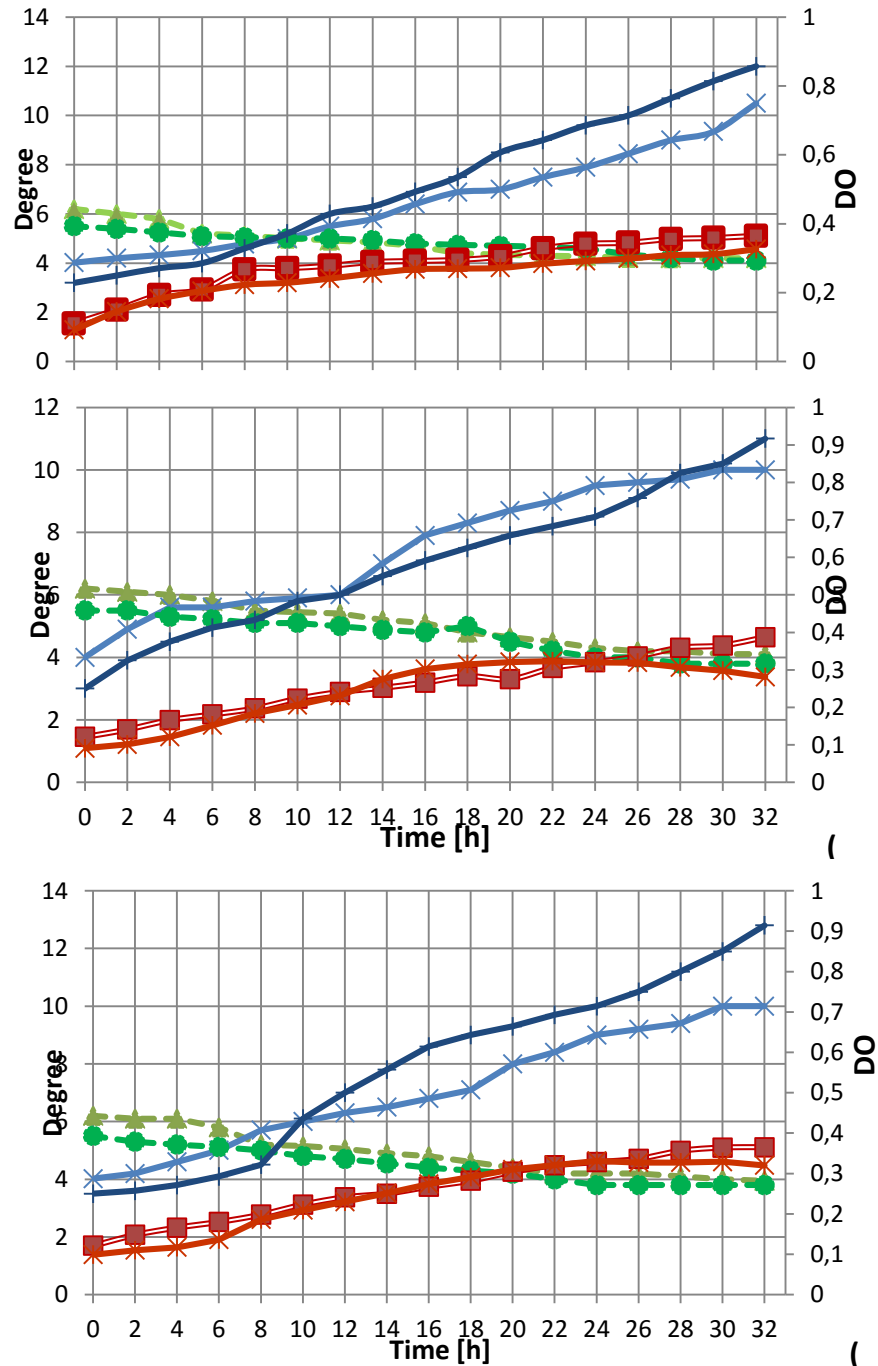


Figure 37. The growth kinetics of selected *Lactobacillus*., namely, *Lactobacillus plantarum* (a), *Enterococcus durans* (b), *Lactobacillus fermentum* (c) cultivated in the liquid fermentation BJFA-MS medium or de Man, Rogosa, and Sharpe (MRS) broth, comparatively. Results are presented as the Optical density.

For *Lactobacillus plantarum*, the BJFA-MS medium demonstrated a slightly higher maximal growth rate (μ_{max}) of 0.015 h^{-1} , compared to 0.014 h^{-1} in the MRS medium. Furthermore, the generation time (G) was shorter in BJFA-MS (46.2 h) than in MRS (49.5 h), indicating a faster rate of bacterial growth. However, a significant difference was observed in the acid production rate (Qac) and productivity. The Qac was higher in BJFA-MS (1.7 g/g.h) compared to MRS (1.03 g/g.h), although the productivity in BJFA-MS was slightly lower (1.75 g/L.h) than in MRS (2.1 g/L.h). Despite this, BJFA-MS exhibited comparable, or even slightly improved, performance in supporting *L. plantarum* fermentation, suggesting its potential as a substitute for MRS medium, particularly due to its similar growth parameters and enhanced acid production.

For *Enterococcus durans*, the comparison between BJFA-MS and MRS media revealed significant differences in bacterial growth parameters. BJFA-MS exhibited a much higher maximal growth rate (μ_{max}) of 0.074 h^{-1} , compared to just 0.028 h^{-1} in MRS. The generation time was notably shorter in BJFA-MS (9.37 h) than in MRS (24.57 h), demonstrating significantly faster bacterial growth. Additionally, the acid production rate (Qac) and productivity were both considerably higher in BJFA-MS (3.82 g/g.h and 1.87 g/L.h , respectively) than in MRS (2.5 g/g.h and 2.53 g/L.h , respectively). These results (refer to Table 4) highlight the superior performance of BJFA-MS in supporting *E. durans* fermentation, suggesting it could serve as a more effective alternative to MRS medium.

Lastly, for *Lactobacillus fermentum*, BJFA-MS also showed distinct advantages over MRS medium in several growth parameters. BJFA-MS had a higher maximal growth rate (μ_{max}) of 0.05 h^{-1} , compared to 0.034 h^{-1} in MRS. The generation time was significantly shorter in BJFA-MS (13.9 h) than in MRS (20.39 h), indicating a faster growth rate. Furthermore, both the acid production rate (Qac) and productivity were substantially higher in BJFA-MS (4.05 g/g.h and 1.8 g/L.h , respectively) compared to MRS (1.85 g/g.h and 2.1 g/L.h , respectively). These findings suggest that BJFA-MS outperforms MRS medium in supporting *L. fermentum* fermentation, emphasizing its potential as a more efficient alternative.

Table 13. Growth parameters of selected Lactic strain cultivated in MRS and the BJFA-MS medium

Parameter ¹	$\mu_{max} \text{ (h}^{-1}\text{)}$		G (h)		Qac (g/g.h)		Productivity (g/L.h)	
	MRS ²	BJFA-MA ³	MRS	BJFA-MA	MRS	BJFA-MA	MRS	BJFA-MA
<i>L. plantarum</i>	0.014	0.015	49.5	46.2	1.03	1.7	2.1	1.75
<i>E. durans</i>	0.028	0.074	24.57	9.37	2.5	3.82	2.53	1.87

<i>L. fermentum</i>	0.034	0.05	20.39	13.9	1.85	4.05	2.1	1.8
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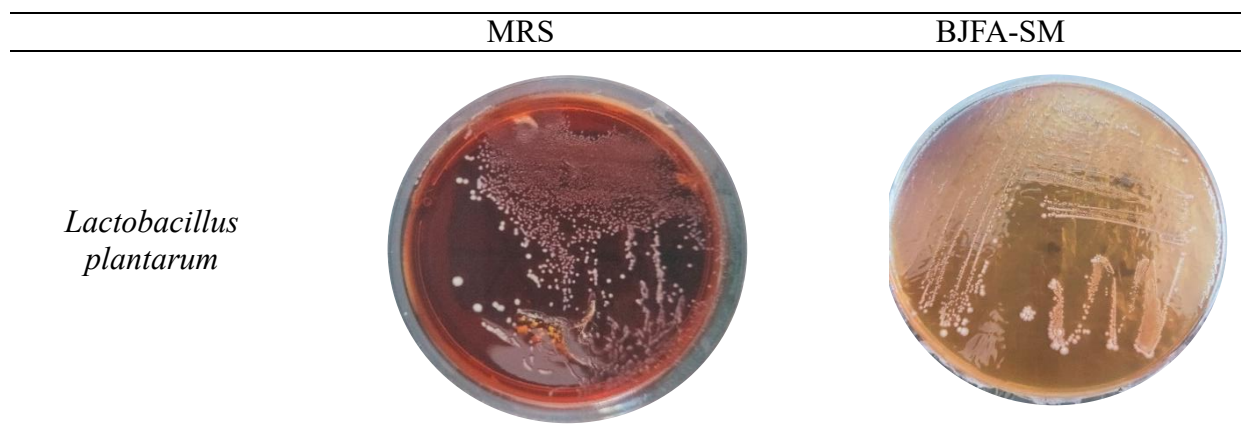
1 Analysed parameters: μ_{\max} (h^{-1})—maximum growth rate; G (h)—generation time; Qac (g/g.h) —Specific acid production rate in grams of acid formed per gram; Productivity (g/L.h)—the quantity of acid formed (g/L) per hour (h). 2 MRS is the abbreviation of de Man, Rogosa, and Sharpe broth; 3 BJFA-MS stand for the Beetroot Juice Fixed Additives-Manganese Sulfate.

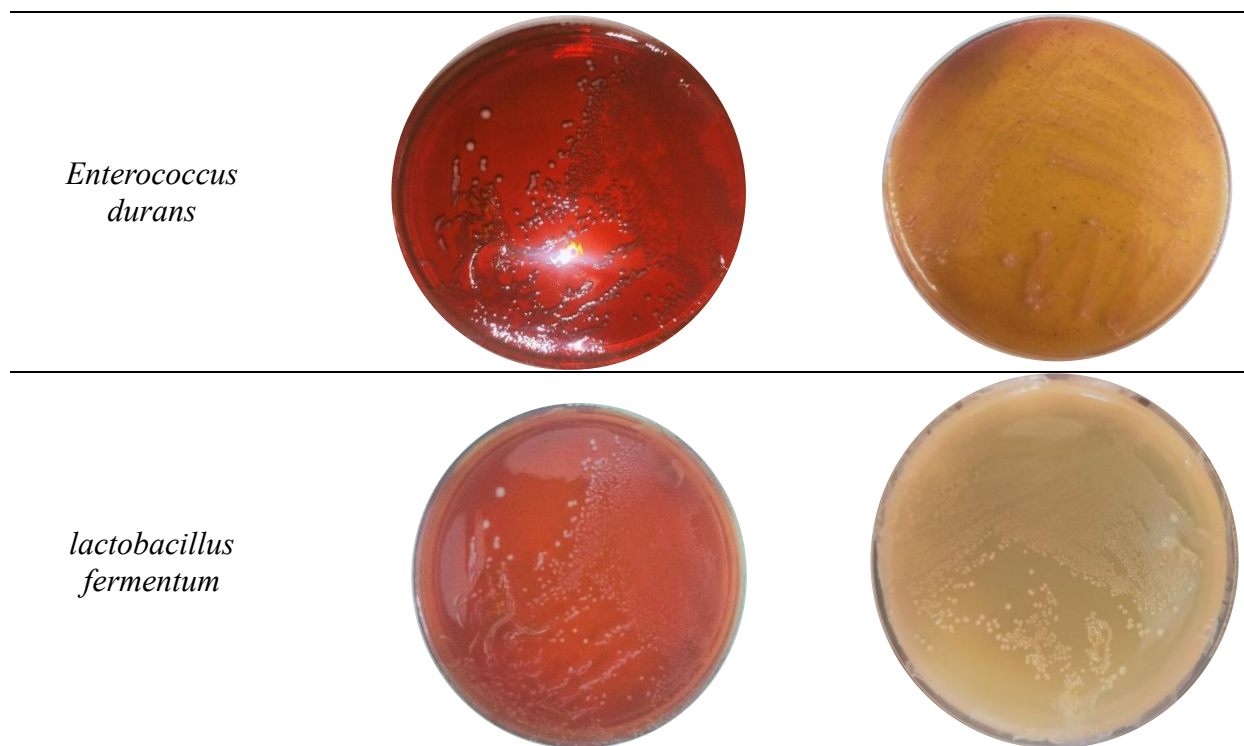
2.7. Agar plate growth

Lactic acid bacteria (LAB) require a complex array of nutrients, including carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, and vitamins (Hébert et al., 2009). Among these, the acquisition of amino acids is particularly challenging for LAB. To address this, chemically defined media have been developed to satisfy their precise nutritional needs. A similar study conducted by Juliana et al. (2010) evaluated cane molasses as a substrate for *Lactobacillus plantarum* growth. Their findings suggest that cane molasses could serve as a cost-effective alternative to importing strains, offering economic benefits to consumers and the Colombian industry (Ossa et al., 2010). Building on this, our experimental framework expanded to include specific chemical agents such as magnesium sulfate, manganese sulfate, sodium acetate, ammonium citrate, di-potassium phosphate, and zinc oxide, which were integrated into the beetroot and additive mixture. This comprehensive approach aimed to investigate the intricate interactions among these components, exploring the potential of beetroot-derived media to support LAB growth. These findings could have significant implications for replacing traditional MRS medium with beetroot-based alternatives.

Additionally, our investigation identified that the medium BJFA-MS demonstrated superior performance for all three lactic acid bacteria strains examined. This formulation was subsequently selected for agar plate testing. Encouragingly, the results from the agar plate testing revealed promising outcomes when compared to the conventional MRS medium. The referred medium exhibited comparable macroscopic traits of cultivation those observed with the MRS medium.

Table 14. Agar plating method for the medium BJFA-MS





Discussion of first-stage results

Our endeavor on fermenting beetroot with lactic acid bacteria (LAB) could mark a pivotal stepping stone in exploring alternative growth media for LAB. The results of the physicochemical analysis reveal a pH value of 5.5, congruent with previous research, the substantial titratable acidity of 13% in beet juice highlights its rich organic acid content. Moreover, the sugar concentration within the beet-based medium is considered suitable and a favorable substrate for LAB growth. The fermentation outcomes shed light on the impotence of medium composition in shaping LAB growth dynamics since they are exigent bacteria.

While beetroot juice alone expressed limited growth, however it was not a negative result which helped us consolidate our basic hypothesis and build on it. When auditioned enriched with add-ins, the formulations demonstrated enhanced bacterial proliferation, emphasizing the significance of nutritional supplementation. Particularly noteworthy was the formulation featuring manganese sulphate (BJFA-MS), which emerged as a promising contender to MRS medium, displaying comparable bacterial growth and macroscopic characteristics.

Further analysis of the kinetic criteria unraveled the potency of various media in promoting LAB growth. While some formulations exhibited higher specific growth rates, a more detailed examination of generation time underscored the comparable efficiency of BJFA-MS, particularly for strains such as *L. fermentum* and *E. durans*.

When comparing the growth kinetics of *Lactobacillus plantarum* across different media formulations to the standard MRS medium, distinct patterns emerge. Beetroot alone resulted in a considerably slower growth rate, with a higher maximum generation time, implying possible nutrient limitations. In contrast, BJFA-PP and BJFA-MG exhibited growth rates similar to or slightly better than MRS. However, BJFA-MS stood out with markedly faster growth kinetics, suggesting it provides a more favorable environment for bacterial proliferation. These results are contrastable to the finding of Utami et al., 2010 where the maximum growth rate was found to be 0.17 h⁻¹ which is higher than our own finding (Yoyok, Budi, Pramono., Eni, Harmayani., Tyas, 2010) This comparison can be linked to variations in cultural conditions, as the genus used may exhibit superior performance due to its enzymatic capabilities. Nevertheless, this outcome is still considered positive, as our findings fall within the favorable spectrum.

In the second, when contrasting the growth kinetics of *Enterococcus durans* across various formulations to MRS medium, we find that for Beetroot alone exhibited a significantly shorter maximum generation time of 0.091, compared to MRS's 50.22, indicating faster growth. Likewise, in the BJFA-MS, BJFA-MG, BJFA-ZO, and BJFA-PP formulations, *Enterococcus durans* demonstrated notably shorter maximum generation times (0.073, 0.112, 0.109, and 0.031, respectively) when compared to MRS. These findings collectively underscore the potential of beetroot-based media in promoting faster growth rates for *Enterococcus durans*, indicating their viability as alternatives to conventional MRS medium. Such outcomes hint at the possibility of harnessing beetroot-derived formulations to optimize microbial growth conditions, offering sustainable and efficient avenues for microbial cultivation. Finally, the case *Lactobacillus fermentum* growth kinetics across various media formulations against the MRS reference, notable differences emerge. Beetroot alone demonstrated a substantially reduced maximum generation time (15.75 hours), suggesting its potential as a growth-enhancing medium. Conversely, the BJFA-ZO medium exhibited a significantly prolonged maximum generation time (77.01 hours), indicating potential growth inhibition with zinc oxide inclusion. While BJFA-SM and BJFA-MG showed comparable generation times to MRS, the BJFA-PP medium displayed a slightly shortened generation time (27.72 hours), hinting at potassium phosphate's mild growth-promoting effect. These variations underscore the sensitivity of *Lactobacillus fermentum* to medium composition, emphasizing the need for nuanced formulation strategies to optimize growth conditions.

The Color-metrics analysis provided additional clarity regarding the transformative effects of LAB fermentation on beetroot juice. Substantial changes in color indexes, including an increase in Yellow* value and a reduction in the Red* value, underscored the metabolic activity of LAB and its influence on pigment composition. These alterations, attributed to Lactic acid formation and pH reduction, highlight the potential of LAB-mediated fermentation in enhancing the nutritional and sensory properties of beetroot-based products. The agar plating results serve as a conclusive validation of BJFA-MS as a conducive growth medium for LAB, showcasing comparable performance to MRS medium. This not only reaffirms the feasibility of beetroot-derived alternatives but also underscores their potential to offer cost-effective and sustainable solutions for

LAB cultivation. Overall, the study opens new avenues for exploration in LAB cultivation and underscores the potential of beetroot-derived mediums in revolutionizing the fields.

Second stage Results

2.8. Physicochemical Analysis

The results of the physicochemical analysis are presented in Figure.31.

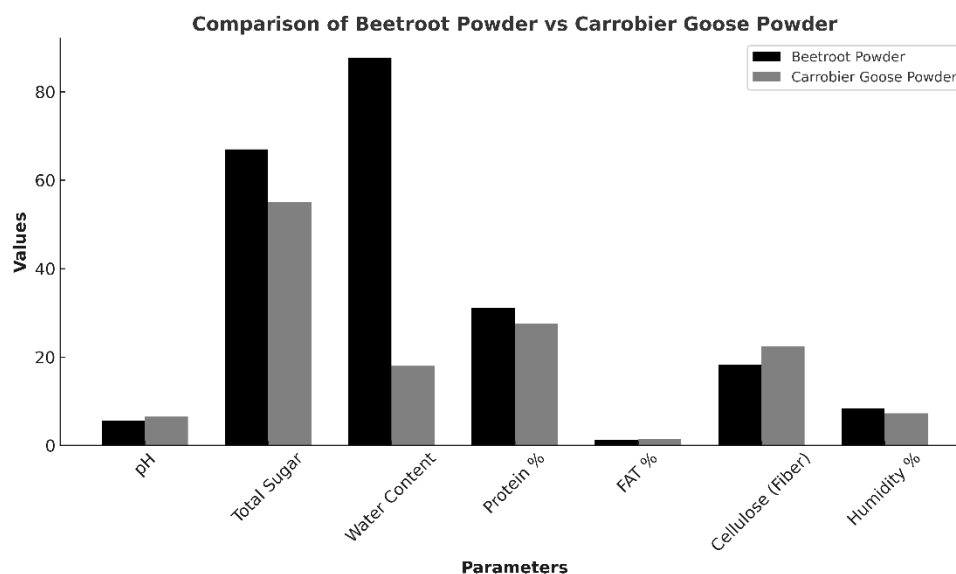


Figure 38. result of physicochemical analysis of beetroot pulp and Carob

2.9. Fermentation

Fermenting beetroot juice with *Lactobacillus plantarum* has proven effective in supporting the growth of various lactic acid bacteria, suggesting its potential as a non-dairy probiotic product. In our study, *L. plantarum*, *L. fermentum*, and *E. durans* were fermented in beetroot juice alone and with various formulations, referred to as BA (beetroot juice Alone); CA (Carob Alone); CBB (Carob Beetroot Blend) and BJP (Beetroot Juice added Peptone). The initial inoculum was set at 10^8 log CFU/mL, and bacterial growth was measured through optical density and (Fig. 2,3.4) represent the results of the three LAB fermentation in this different medium. While beetroot juice alone resulted in limited growth, likely due to its insufficient nutritional content, supplementation with meat extract, yeast extract, peptone led to significant growth across all strains. Additionally, following a similar growth trajectory. These findings support earlier studies, including Muneeb et al. (2019).who observed similar growth patterns in *L. plantarum* when fermenting beetroot juice, and Wang et al. (2015), who demonstrated that beetroot contains the necessary nutrients for LAB growth and lactic acid production during fermentation (Machulin et al., 2022; Malik et al., 2019b; Wang, Na., Xiong, Guoxi., Si, Hui., Wang, Ping., Wang, 2015). Refer to Figure. 32,33.31

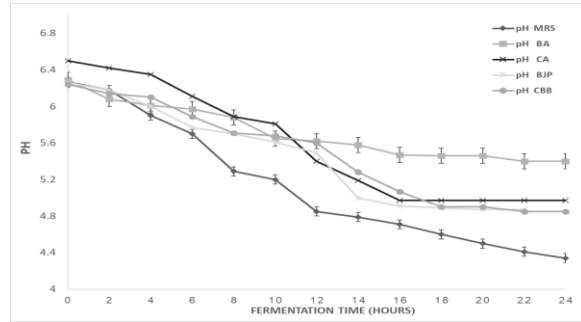
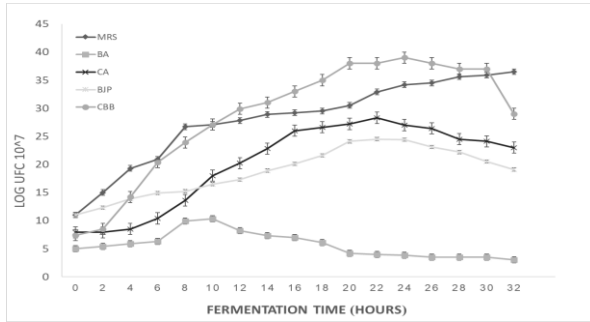


Figure 39. OD (Optical Density) values, LoG UFC ml⁻¹(Unit Forming Colony), pH and Acidity of Strain: *Enterococcus durans* . in different growth medium. a: LAB viable counts; b pH; c Total titrable acidity. The experimental data were fitted to the logistic model. All treatment were performed in triplicate

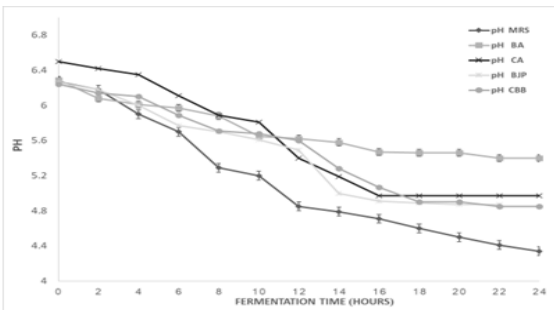
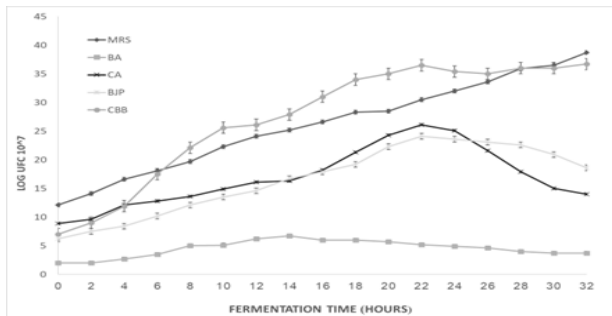
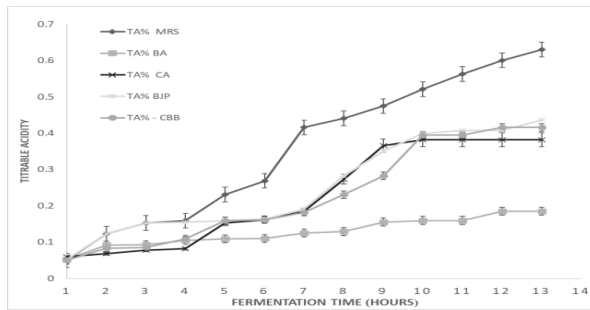
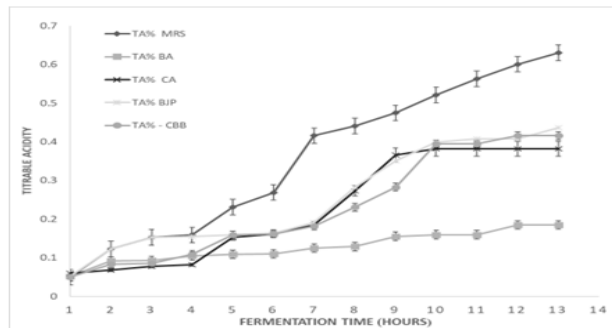


Figure 40. OD (Optical Density) values, LoG UFC ml⁻¹(Unit Forming Colony), pH and Titrable Acidity of Strain: *Lactobacillus plantarum* ; a were fitted to the logistic model. All treatment were performed in triplicate



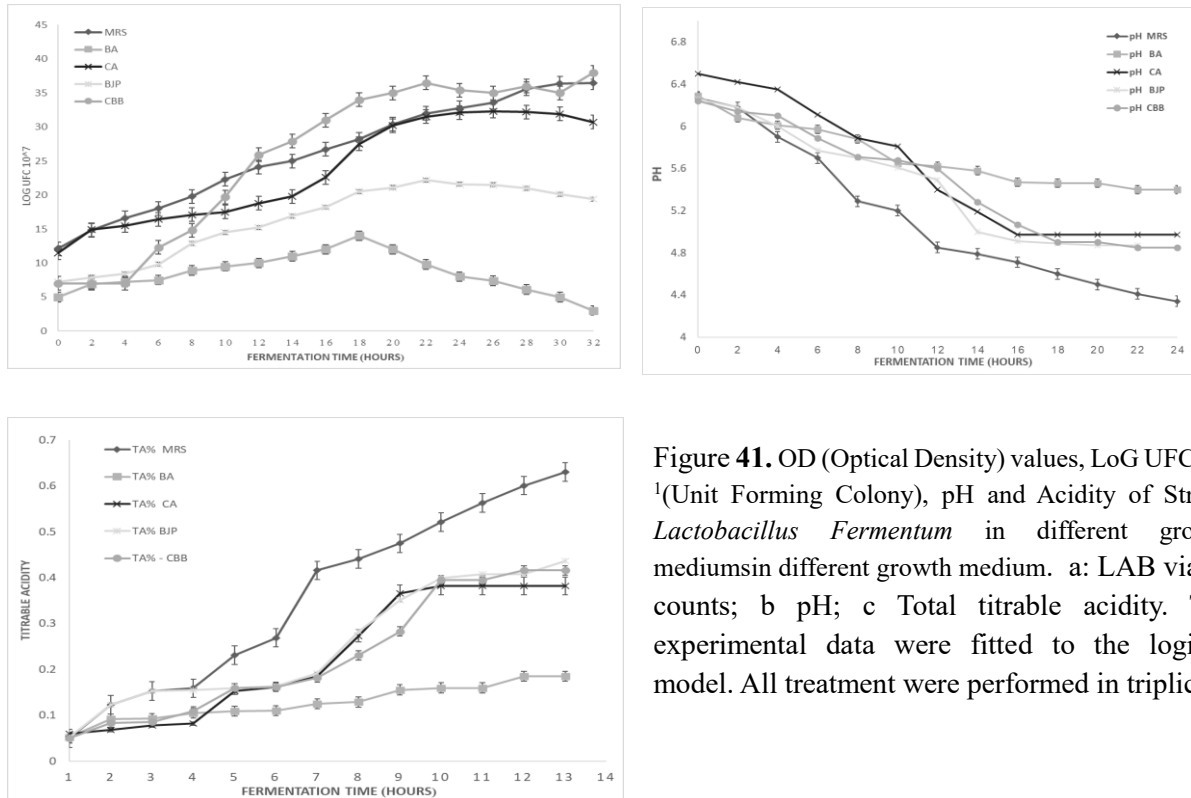


Figure 41. OD (Optical Density) values, LoG UFC ml⁻¹(Unit Forming Colony), pH and Acidity of Strain: *Lactobacillus Fermentum* in different growth medium. a: LAB viable counts; b pH; c Total titrable acidity. The experimental data were fitted to the logistic model. All treatment were performed in triplicate

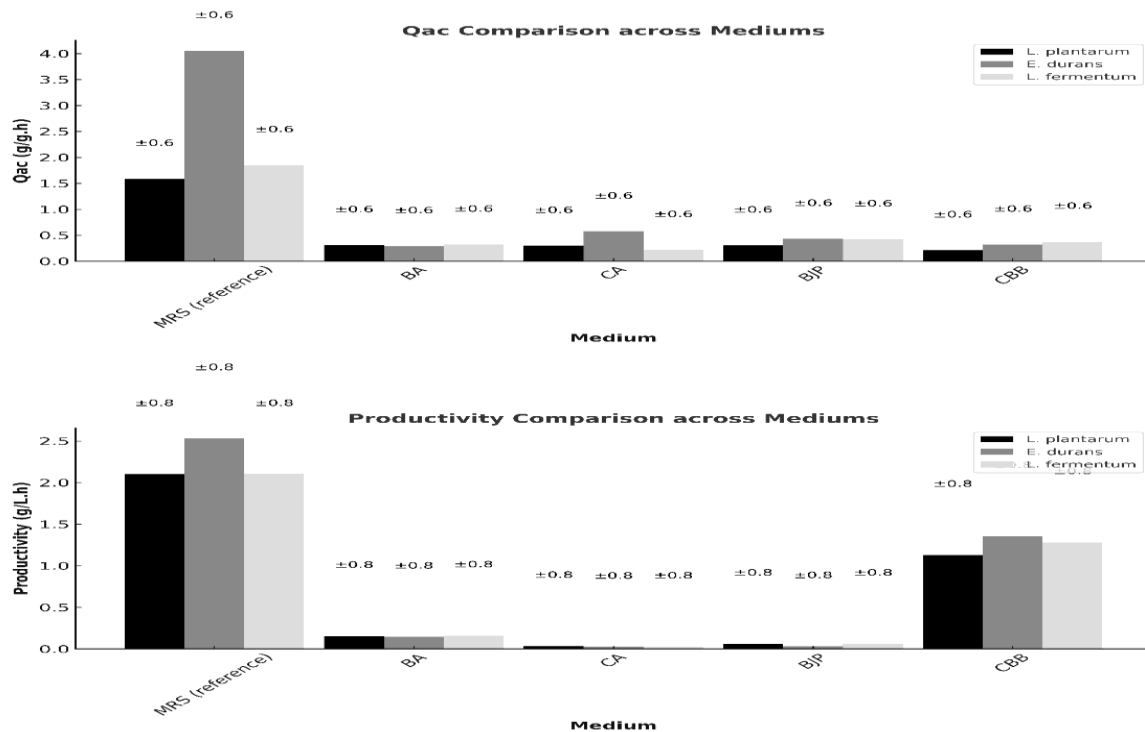


Figure 42: OD (Optical Density) values, LoG UFC ml⁻¹(Unit Forming Colony), pH and Acidity of Strain: *Lactobacillus Fermentum* in different growth mediums. a: LAB viable counts; b pH; c Total titrable acidity. The experimental data were fitted to the logistic mode. All treatment were performed in triplicate.

2.10. Kinetic findings

Since the establishment of MRS medium, it has been the standard for cultivating and propagating lactic acid bacteria. However, there is a growing need to diversify the mediums used for growing these beneficial bacteria to enable further study and applications. In this context, our study focused on developing a semi-natural medium from beetroot pulp, incorporating the minimum nutritional requirements necessary for lactic acid bacteria, which are known for their specific trophic needs. We calculated the specific growth rate (μ_{max}) and generation time for each medium using data from the fermentation process, results are represented in Fig.30. While several formulations from the BJ series exhibited higher growth rates, it is crucial to interpret the growth speed alongside generation time for a proper comparison to the reference MRS medium.

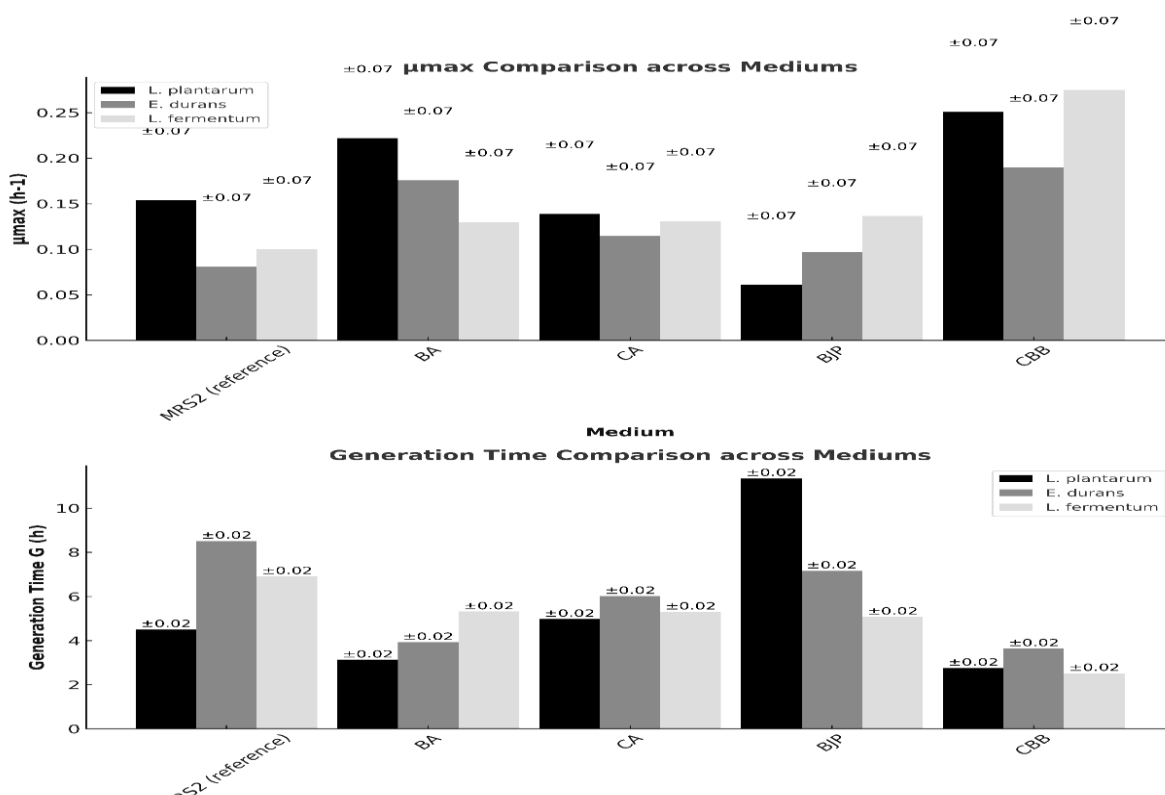


Figure 43: Growth parameters of selected Lactic strain cultivated in MRS, CBB and the BJP medium. Qac: Specific acid production rate in grams of acid formed per gram; Productivity (g/L.h)—the quantity of acid formed (g/L) per hour (h); MRS is the abbreviation of de Man, Rogosa, and Sharpe broth; BA stands for the Beetroot Juice Alone; CA stands for the Carrober pods Alone; BJA stands for the Beetroot Juice with added peptone ;CBB stands for Carrober ,Beetroot Juice Blend *the findings are shown as mean \pm standard deviation (S.D).


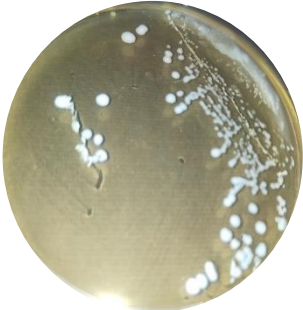




2.11. Productivity and Qac

The production and Specific acid production *Qac* are calculated for each medium using the collected data, and expressed in Figure 36.

2.12. Agar plate growth

Results of the bacterial culture on the formulated medium that expressed significant performance from the result of the fermentation are represented in Table 15.

Table 15. The growth result of the formulated medium that exhibited Lactic acid bacterial growth

medium	Cultural aspect		
	<i>L.plantarum</i>	<i>E. durans</i>	<i>L. Fermentum</i>
MRS ¹			
BJP ²			

CBB³



¹: Man De Sharp Rogusa medium, ²: Beetroot Juice with added peptone; ³: Carrobier Beetroot Blend.

2.13. Statistical test results

The analysis began with an ANOVA (Single Factor) test to evaluate significant differences in key parameters— μ_{max} , Generation Time, Qac, and Productivity across various media types. The results, marked by an F-value of 99.78 and a p-value of 0.00, indicated significant differences, prompting a detailed examination through the Turkey post-hoc test. The test showed that media CBB outperformed BA and CA in several parameters and was comparable to MRS medium. Correlation analysis revealed strong relationships, especially between Qac and productivity across *L. plantarum*, *E. durans*, and *L. fermentum*. A weighted scoring system confirmed BJP and CBB as the top-performing media for all bacteria, indicating superior efficacy.

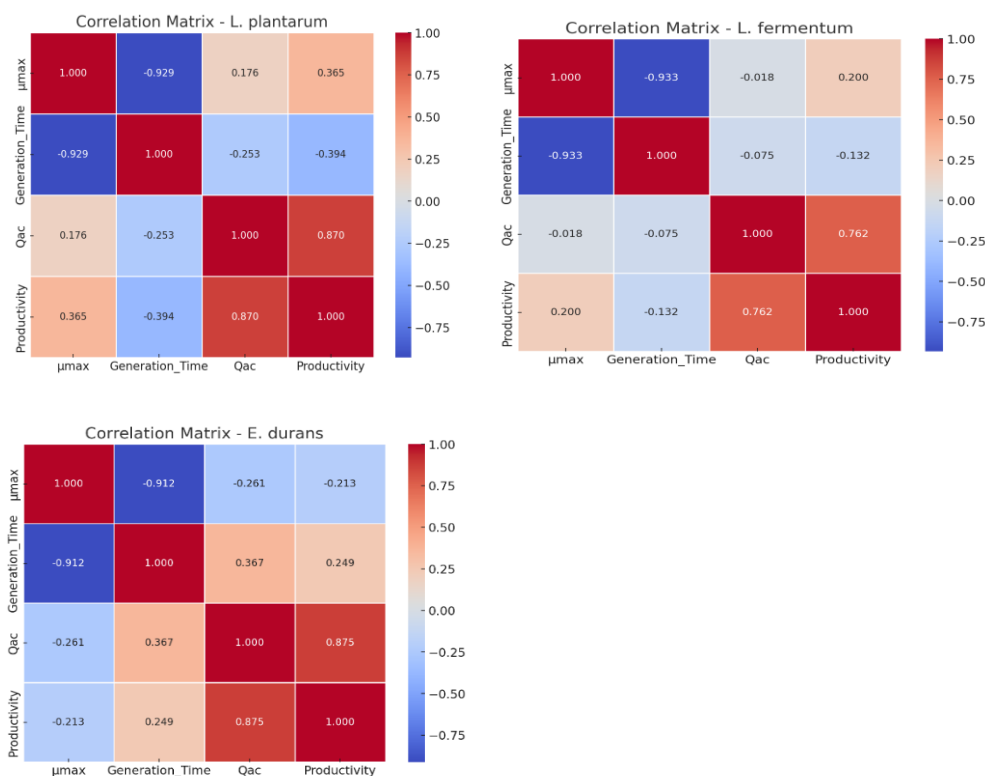


Figure 44:Representation of the statistical Result output in a heatmap for all tested Strains: *L. plantarum*, *E. durans* and *L. fermentum*. *The color intensity represents the strength and direction of the correlations, with blue indicating negative correlations and red indicating positive correlations.

Discussion of second-stage results

The primary aim of this study was to validate the feasibility of a BA-based medium and its alternative formulations for supporting the growth of *Lactobacillus plantarum*, *Enterococcus durans*, and *Lactobacillus fermentum*. Unlike previous research, which mainly focused on fermentation and probiotic effects in food, our study concentrated on developing a plant-based growth medium to replace the widely used MRS medium (Hébert et al., 2009). LAB's nutritional needs, including carbohydrates, amino acids, peptides, fatty acids, and vitamins, were addressed in a BA-derived medium enriched with Carob to optimize growth. Previous studies have isolated LAB from BA products, with strains like *Lactobacillus plantarum* thriving in BA juice without extra nutrients, supporting BA as a viable alternative to traditional media like MRS.

Our findings revealed that the enriched BA formulations, particularly the blend containing CA (Carrobier) pod ground powder, supported LAB growth comparable to MRS, indicating the potential of plant-based media for LAB cultivation. The formulation containing CA significantly enhanced the growth rate of LAB, with the highest μ_{max} observed in the CBB medium. In contrast, BA alone exhibited slower growth, confirming the necessity of supplementation for optimal LAB proliferation. These results align with previous studies, such as that of Kyung et al. (Kyung et al., 2005), which demonstrated that LAB can thrive in plant-based media under suitable conditions.

Furthermore, earlier research has highlighted the potential of isolating LAB from beetroot products. For instance, *Lactococcus lactis* and *Weissella cibaria* strains were successfully isolated from homemade fermented beetroot. Panghal et al. (2017) found that LAB species such as *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Lactobacillus delbrueckii* survived on pasteurized BA juice without extra nutrients but at a short rate. Malik et al. (2019) showed that *Lactobacillus casei*, *L. plantarum*, and *L. acidophilus* grew in carrot and BA juice substrates, producing lactic acid after 48 hours of fermentation at 35°C (Malik et al., 2019a).

Growth performance and productivity analyses revealed that CBB exhibited the highest growth rates for *L. plantarum* (0.251 h^{-1}) and *L. fermentum* (0.275 h^{-1}), surpassing the MRS medium, validating CBB as an effective medium for LAB cultivation. The generation time for *L. plantarum* was shortest in CBB (2.760 h), confirming its superior growth-supporting properties. Similarly, specific acid production rates and productivity were highest in CBB, highlighting its potential as a sustainable and efficient alternative for LAB cultivation. In comparative analysis, CBB outperformed MRS and other formulations, indicating that CA extract enhances LAB metabolism. The BA and CA combination promoted superior growth and acid production, eliminating the need for peptone supplementation. Additionally, these media effectively inhibited non-LAB microorganisms, confirming plant-based media as sustainable alternatives to traditional ones.

2.14. Growth Potential and Medium Formulation

In our study, a decrease in pH was a key indicator of successful fermentation, with notable differences in acid production and pH reduction across various media formulations. Specifically, carob- and soybean-enriched formulations showed greater potential for enhancing LAB growth and acidification compared to the more limited outcomes observed in Kyung et al.'s beet juice fermentation. This suggests that our media modifications provide a more robust alternative for LAB proliferation. Unlike Kyung et al. (2005), who observed minimal changes in viable cell counts after 48 hours of fermentation, our formulations showed different growth patterns based on medium composition. While Kyung's study found only *Lactobacillus acidophilus* and *L. plantarum* significantly lowering beet juice pH, our study showed *L. plantarum*, *L. fermentum*, and *E. durans* also acidifying the medium, demonstrating their ability to thrive in plant-based media.

We examined the growth potential of BA, CA, and soybean individually, confirming that these plant-based media could support LAB growth, even at minimal levels. The maximum specific growth rates (μ_{max}) were 0.22 for BA, 0.139 for CA, and 0.095 for soybean, with corresponding generation times of 3.9, 6.0, and 7.1 hours, respectively. These results align with the speculation by Tuorila and Cardello (2002) (Tuorila & Gardello, 2002). Their research suggested that fruit and vegetable juices could serve as viable media for probiotic growth. In our study, *L. plantarum*, *L. fermentum*, and *E. durans* showed varying degrees of acidification, further highlighting their potential to thrive in plant-based formulations. This suggests that plant-based media, such as BA, CA, and soybean, hold promise for sustaining LAB growth even at relatively low levels.

The comparative evaluation of media formulations highlighted the superior performance of CBB in supporting the growth of *L. plantarum* and *L. fermentum*, excelling in both growth rate and acid production. While MRS medium remained a reliable reference, the CBB formulation outperformed it, indicating that the combination of BA and CA extracts provides essential nutrients that enhance LAB metabolism. A key finding of this study was the comparison between CBB and BJP, which revealed that CA extract effectively replaces peptone supplementation, resulting in higher bacterial growth. This suggests that the bioactive compounds in CA enhance LAB viability and fermentation efficiency, positioning it as a promising ingredient for biotechnological applications.

2.15. Statistical results

- ***L. plantarum***: The heatmap reveals a strong negative correlation between μ_{max} and Generation Time (-0.929), indicating that as the growth rate increases, the time it takes for the population to double decreases. Additionally, there is a notable positive correlation between Qac and Productivity (0.870), suggesting that higher substrate consumption is associated with higher productivity. However, the relationship between μ_{max} and Qac is

weakly positive (0.176), implying that while growth rate slightly influences substrate consumption, the connection is not as strong.

- **L. fermentum:** In *L. fermentum*, the heatmap also shows a significant negative correlation between μ_{\max} and Generation Time (-0.933), consistent with the general trend observed in other strains. The correlation between Qac and Productivity (0.762) is strong, further emphasizing that higher substrate consumption leads to increased productivity. The relationship between μ_{\max} and Productivity is weak but positive (0.200), suggesting a minor connection between the growth rate and productivity, although it is not as pronounced as with Qac.
- **E. durans:** The heatmap shows a strong negative correlation between μ_{\max} and Generation Time (-0.912), mirroring the findings in *L. plantarum* and *L. fermentum*. There is a very strong positive correlation between Qac and Productivity (0.875), indicating a clear relationship between higher substrate consumption and greater productivity. Interestingly, μ_{\max} and Productivity exhibit a weak negative correlation (-0.213), suggesting that in this strain, faster growth does not strongly influence productivity and might even slightly hinder it. Each strain exhibits a general pattern where μ_{\max} and Generation Time are negatively correlated, and Qac and Productivity are positively correlated. However, there are differences in the strength of relationships among other parameters across strains.

Third stage Results

3.1. Physicochemical analysis

The chemical analyses of beetroot and soybean powders revealed distinct compositional characteristics, as summarized in Figure 1. Beetroot powder had a slightly acidic pH of 5.6, whereas soybean powder exhibited a pH of 6.2, consistent with findings by Sakhare et al. (Sakhare et al., 2019) and slightly higher than the typical 6.5-7.0 range reported by Osthoff et al. (Osthoff et al., 2010) Total sugar content was substantial in both powders, at 67% for beetroot and 70.2% for soybean, aligning with the generally high carbohydrate content of soybeans, though some studies indicate lower sugar levels. (Głowacka et al., 2019) Water content was markedly higher in beetroot powder (87.6%) compared to soybean powder (63.1%), reflecting beetroot's higher moisture retention capability, as also noted by (Sakhare et al., 2019) and supported by other studies highlighting soybean's comparatively lower moisture retention. (Głowacka et al., 2019; Wang et al., 2021) Protein content was significantly greater in soybean powder (41.67%) than beetroot powder (31.09%), aligning with soybean's known high protein composition, (Chen et al., 2021a) though some beetroot varieties have reported slightly higher protein contents. Similarly, fat content was substantially higher in soybean powder (14.89%) compared to beetroot powder (1.32%), consistent with soybeans' lipid-rich profile, whereas beetroot is recognized as having negligible fat content. (Krogh et al., 2015) Fiber content was notably greater in beetroot powder (18.32% cellulose) than soybean powder (2%), reflecting beetroot's higher dietary fiber content compared to the typically lower fiber levels in soybeans. (Flores-Mancha et al., 2021) Lastly, moisture levels were higher in beetroot powder (8.45%) relative to soybean powder (1.38%), consistent with beetroot's higher water activity and soybean's lower moisture retention capacity (Figure 38). (Carmo et al., 2018)

The primary objective of this study was to assess the viability of lactic acid bacteria (LAB), specifically *Lactobacillus plantarum*, *Enterococcus durans* and *Lactobacillus fermentum*, in beetroot-based media and their formulations as sustainable alternatives to the conventional MRS medium. Previous studies exploring similar objectives frequently focused on fermentation or probiotic effects in food products like yogurt or biscuits; in contrast, our research emphasized optimizing plant-based growth media. Prior investigations affirm the ability of LAB to grow successfully on plant-derived substrates, such as beetroot, with *Lactococcus lactis* and *Weissella cibaria* strains isolated from fermented beetroot products and *Lactobacillus* species demonstrating viability in pasteurized beetroot juice without nutrient supplementation. These findings support our observations, confirming beet juice as a suitable substrate for basic LAB cultivation, particularly for *L. plantarum* and *L. fermentum*. However, as highlighted by Kyung et al., beetroots alone might not offer optimal growth conditions for LAB, as demonstrated by the moderate growth of *Lactobacillus acidophilus* and *L. plantarum* in beetroot juice alone. Our findings corroborate this, indicating that soybean supplementation substantially enhances bacterial proliferation.

Our study demonstrates that a soybean-beetroot blend (SBB) supports bacterial growth comparable to the MRS medium, contrasting with previous studies where plant-based media underperformed traditional formulations. Notably, growth parameters such as μ_{max} and generation time improved significantly in the SBB medium, underscoring its potential as a viable alternative to Man Rogosa Shape. Although *L. plantarum* exhibited robust growth in this formulation, overall acid production and productivity remained inferior to MRS, which consistently promoted superior LAB growth. This discrepancy underscores the necessity for further optimization of the soybean-beetroot medium to enhance LAB proliferation and acid production effectively.

3.2. Fermentation

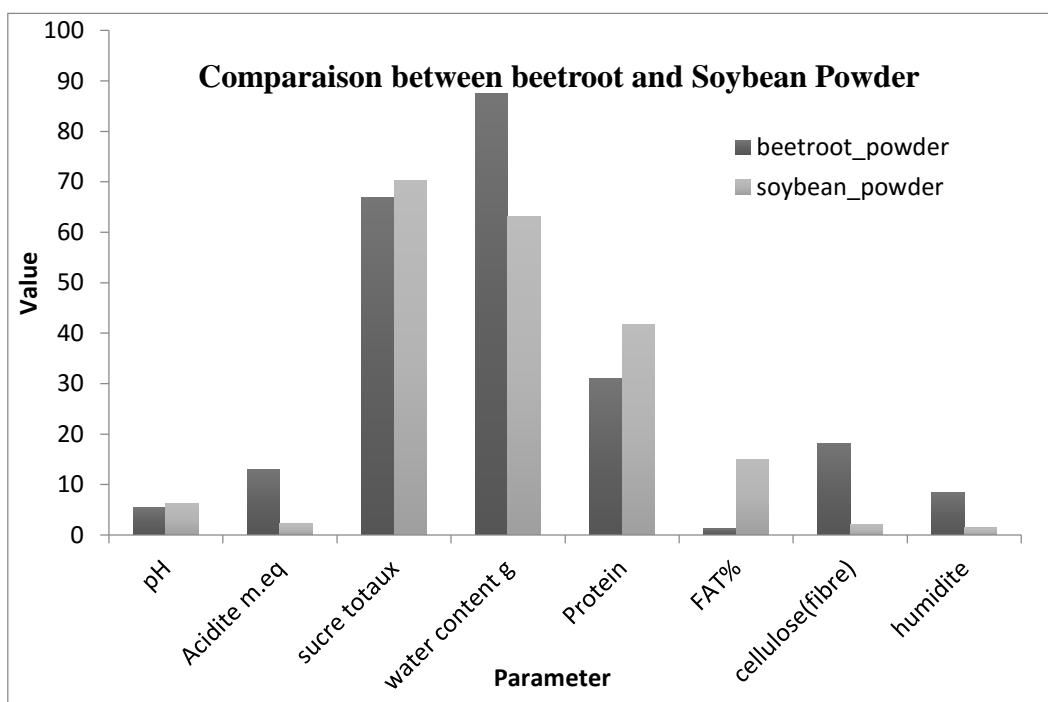


Figure 45. Chemical analysis of the soybean and beetroot powder

This experiment involved the cultivation of three lactic acid bacteria, namely *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Enterococcus durans*, in soybean and beetroot juice individually, as well as in various synergistic formulations. The initial inoculum of all lactic acid bacteria in fermented juices was calibrated at 10^7 logs CFU/mL with optical density serving as an indicator of bacterial growth and medium consumption (Figures 34, 35, 36). Multiple contrasts were observed between soybean juice and beetroot juice, whether alone or with the mixed formulation.

Beetroot juice alone exhibited the lowest growth among the three lactic acid strains throughout the fermentation period. This may be attributed to its limited nutritional value and the high nutritional

requirements of lactic acid bacteria, which are known to be fastidious in their trophic requirements. Slightly improved bacterial growth was observed when beetroot juice was supplemented with meat extract and yeast extract. These results are similar to those described by (Malik et al., 2019b). They exhibited observable growth in *L. plantarum*, with a progressive increase in the number of viable cells over time when beetroot juice was used as the fermentation substrate. This is further supported by Wang et al., who successfully fermented beetroot-based media using lactic acid bacteria, demonstrating that adequate nutritional supplementation is essential for the minimal growth requirements of these microorganisms. (Wang et al., 2015)

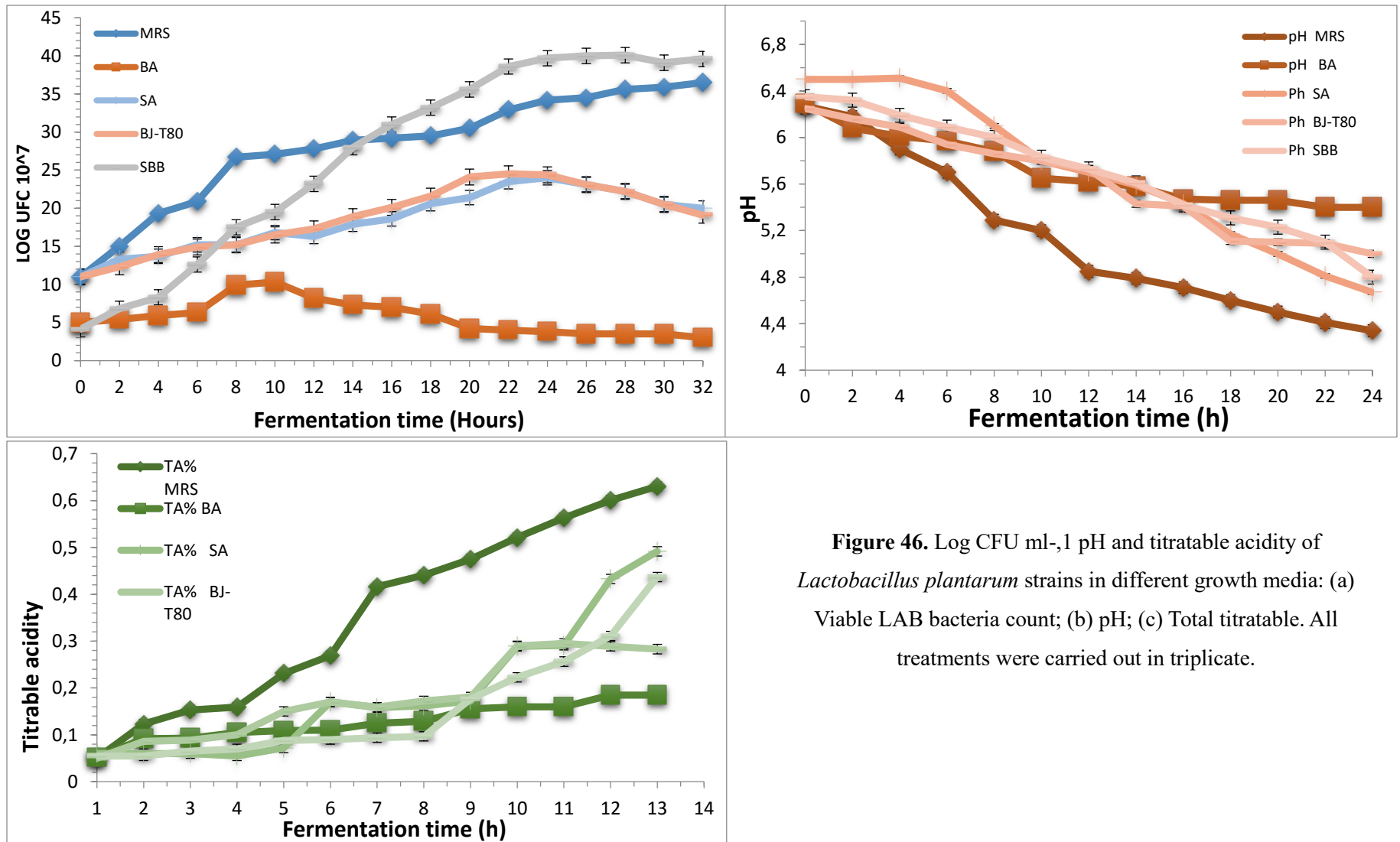


Figure 46. Log CFU ml⁻¹, pH and titratable acidity of *Lactobacillus plantarum* strains in different growth media: (a) Viable LAB bacteria count; (b) pH; (c) Total titratable. All treatments were carried out in triplicate.

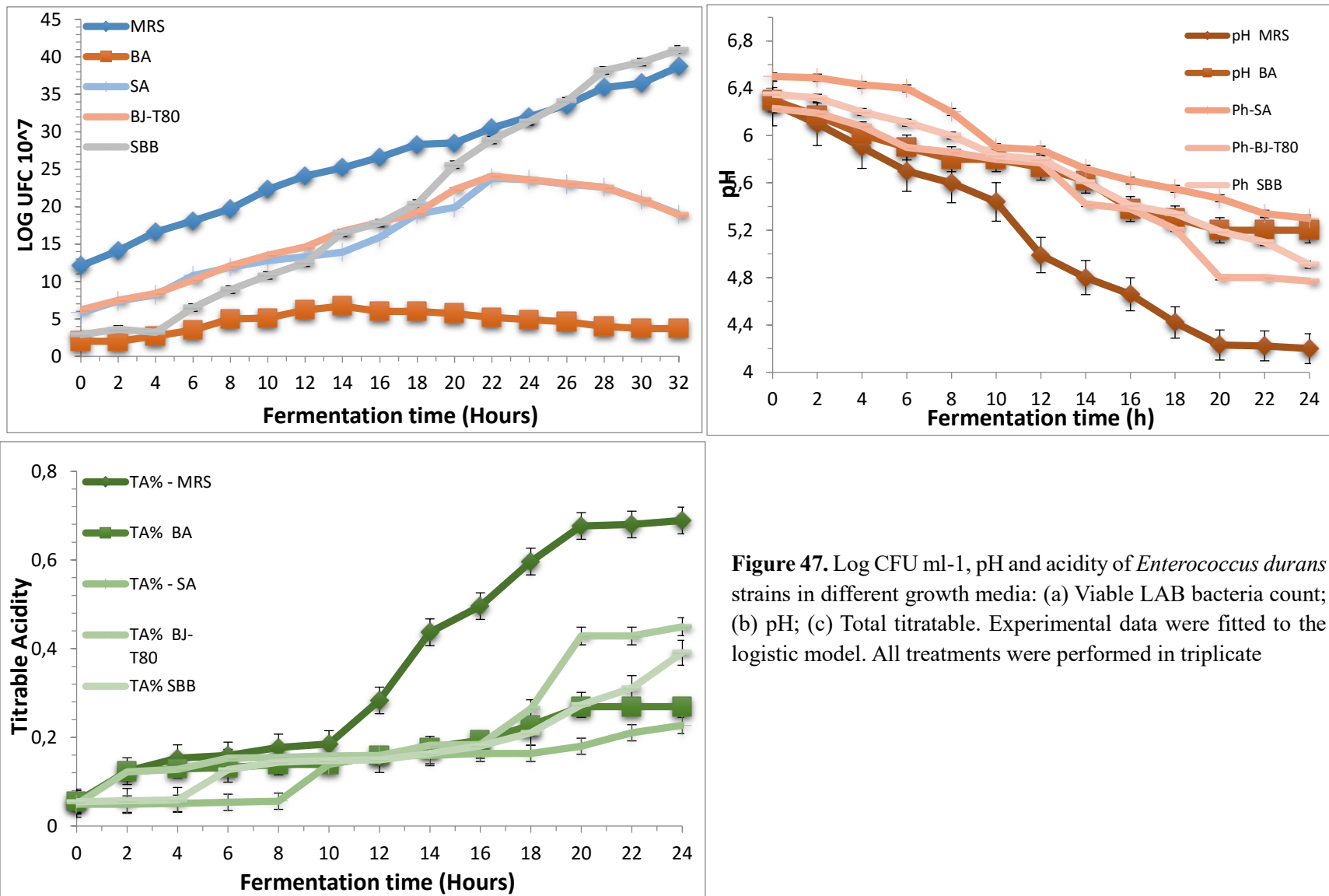


Figure 47. Log CFU ml-1, pH and acidity of *Enterococcus durans* strains in different growth media: (a) Viable LAB bacteria count; (b) pH; (c) Total titratable. Experimental data were fitted to the logistic model. All treatments were performed in triplicate

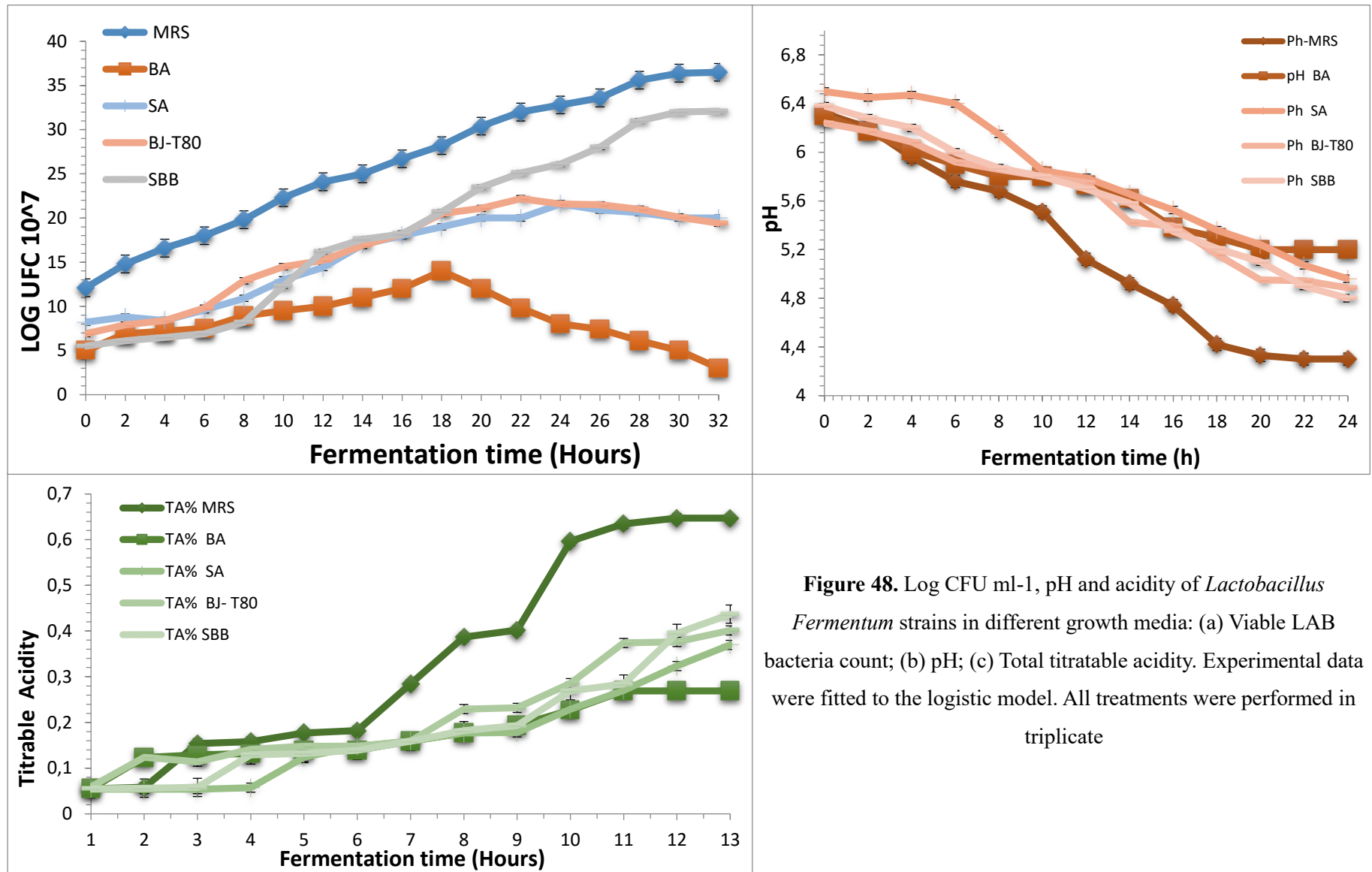


Figure 48. Log CFU ml⁻¹, pH and acidity of *Lactobacillus Fermentum* strains in different growth media: (a) Viable LAB bacteria count; (b) pH; (c) Total titratable acidity. Experimental data were fitted to the logistic model. All treatments were performed in triplicate

3.3. Kinetic Findings

Although MRS medium remains the gold standard for LAB cultivation, increasing interest in sustainable alternatives has driven the development of plant-based media. This study highlights the effectiveness of beetroot-based formulations in promoting LAB growth. Notably, beetroot juice alone resulted in the highest maximum specific growth rate (μ_{max}) for *L. plantarum* (0.222 h^{-1}), surpassing both MRS (0.154 h^{-1}) and soybean-based media. This can be attributed to the abundance of bioactive compounds present in beetroot, such as betalains and flavonoids, (Q. Zhang et al., 2013) supporting findings that emphasize the potential of plant-derived media. (Pérez-Alvarado et al., 2022)

Table 16. Growth parameters of the selected lactic strain cultivated in MRS and the medium

Parameter	Qac (g/g.h)			Productivity (g/L.h)		
	<i>L. plantarum</i>	<i>E. durans</i>	<i>L. fermentum</i>	<i>L. plantarum</i>	<i>E. durans</i>	<i>L. fermentum</i>
MRS ¹	1.588 ± 0.6	4.055 ± 0.6	1.852 ± 0.6	2.100 ± 0.8	2.531 ± 0.8	2.100 ± 0.8
BA ²	0.310 ± 0.6	0.290 ± 0.6	0.320 ± 0.6	0.155 ± 0.8	0.145 ± 0.8	0.160 ± 0.8
SA ³	0.316 ± 0.6	0.336 ± 0.6	0.327 ± 0.6	0.030 ± 0.8	0.025 ± 0.8	0.027 ± 0.8
BJ-T80 ⁴	0.262 ± 0.6	0.536 ± 0.6	0.430 ± 0.6	0.043 ± 0.8	0.061 ± 0.8	0.062 ± 0.8
SBB ⁵	0.161 ± 0.6	0.701 ± 0.6	0.530 ± 0.6	0.028 ± 0.8	0.029 ± 0.8	0.028 ± 0.8

¹: Man, Rogosa, and Sharpe; ²: Beetroot alone; ³: Soybean alone; ⁴: SBB: Stands for soybean-beetroot blend; ⁵: Beetroot juice without Tween 80. The findings are shown as mean ± standard deviation (SD).

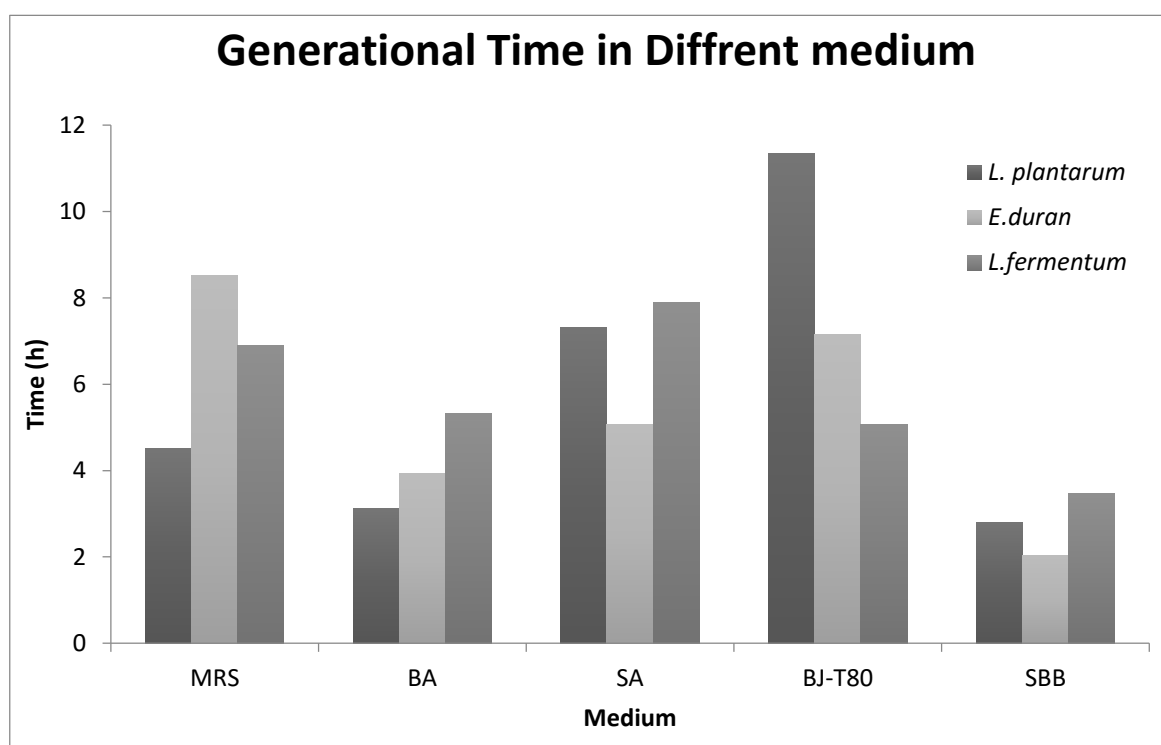


Figure 49. Results of kinetic fermentation of different beetroots with lactic acid bacteria, including MRS (Man, Rogosa and Sharpe), BA (beet alone), SA (soybean alone), SBB (soybean-beetroot mixture) and BJ-T80 (beet juice without Tween

Table 1 summarizes kinetic parameters, showing that *L. plantarum* exhibited the fastest generation time in beetroot juice alone (3.122 h), whereas the soybean-beetroot blend (SBB) formulation emerged as the most efficient medium for *E. durans* (2.037 h), highlighting the advantage of targeted nutrient supplementation. (Quinto et al., 2014) Although BJ-T80 exhibited slower growth, it nonetheless supported LAB proliferation, indicating potential for further medium optimization.

Specific growth parameters (see Figure 5) also confirmed the superior performance of the SBB formulation. For *L. plantarum*, SBB showed a μ_{max} of 0.248 h^{-1} and a generation time of 2.79 hours, while BJ-T80 had lower growth and a higher generation time (μ_{max} of 0.061 h^{-1} and 11 hours, respectively), with MRS medium intermediate between these values (μ_{max} of 0.154 h^{-1} and generation time of 4.5 hours). For *E. durans*, SBB markedly outperformed BJ-T80 (μ_{max} of 0.340 h^{-1} and generation time of 2.037 h), whereas MRS underperformed comparatively (μ_{max} of 0.081 h^{-1}). Similarly, for *L. fermentum*, SBB again excelled (μ_{max} of 0.200 h^{-1}), outperforming BJ-T80 (μ_{max} of 0.137 h^{-1}) and significantly surpassing MRS medium, which showed the lowest efficiency (μ_{max} of 0.100 h^{-1}). These results align with Baygut et al.' who demonstrated that *Lactobacillus acidophilus* effectively fermented a soybean-based beverage, achieving a viable cell count of 4.66 log CFU/g. Furthermore, our study emphasizes that combining soybean and beetroot substrates promotes more effective LAB growth than either component alone.

Our findings underscore the critical role of nutrient supplementation in enhancing LAB performance. Adding nutrients to beetroot and soybean juices significantly boosted LAB growth, particularly in BJ-T80 and SBB formulations compared to individual substrates alone. These observations corroborate Raczyk et al, who reported that tailored supplements enhance LAB fermentation dynamics. Moreover, Sawatari et al. demonstrated that additives like Tween 80 enhance microbial growth in vegetable juices, which was evident in the BJ-T80 formulation. However, even with supplementation, BJ-T80 did not surpass MRS performance, consistent with Sawatari et al. Overall, these findings affirm that optimized beetroot and soybean-based media offer promising eco-friendly alternatives to conventional Man Rogosa Sharpe media, contributing to ongoing efforts to develop sustainable, cost-effective solutions for LAB cultivation in fermentation industries.

3.4. Productivity and QAC

The analysis of acid production rate (Qac) and productivity for selected LAB strains in MRS, beetroot juice-based (BJ-based), and soybean-beetroot blend (SBB) media revealed notable differences (Table 1). MRS medium consistently exhibited superior performance, achieving the highest Qac ($1.588 \text{ g/g}\cdot\text{h}$ for *L. plantarum*) and productivity ($2.10 \text{ g/L}\cdot\text{h}$), confirming its

established efficiency in LAB cultivation.¹¹ Conversely, beetroot alone (BA) and soybean alone (SA) showed significantly lower Qac values (0.310 and 0.316 g/g·h, respectively) and reduced productivity, highlighting their limited capability to support high acid production.

Introducing additives into BJ-T80 slightly improved performance, particularly for *L. plantarum* and *E. durans*, although these enhancements still did not match the performance of MRS. This finding aligns with Quinto et al. who observed that additives could enhance LAB growth but typically yield inferior results compared to traditional media. SBB medium demonstrated better outcomes, particularly for *E. durans* (Qac of 0.701 g/g·h, productivity of 0.029 g/L·h), underscoring the potential of plant-based media when supplemented with appropriate nutrients.

Specifically, for *L. plantarum*, SBB yielded moderate parameters (Qac of 0.161 g/g·h, productivity of 0.028 g/L·h), while BJ-T80 showed improved values (Qac of 0.262 g/g·h, productivity of 0.043 g/L·h); however, MRS still outperformed both. For *E. durans*, BJ-T80 exhibited lower Qac (0.536 g/g·h) but higher productivity (0.061 g/L·h), whereas MRS had the highest performance (Qac of 4.055 g/g·h, productivity of 2.531 g/L·h). Likewise, for *L. fermentum*, SBB recorded a Qac of 0.530 g/g·h and productivity of 0.028 g/L·h, whereas BJ-T80 demonstrated lower Qac (0.430 g/g·h) but higher productivity (0.062 g/L·h), with MRS remaining superior (Qac of 1.852 g/g·h, productivity of 2.100 g/L·h).

These findings highlight the necessity for further optimization of plant-based media to achieve improved acid production and productivity, challenging the prevailing view that MRS universally provides optimal conditions.

3.5. HPLC Results

The HPLC analysis emphasizes the distinct roles of these bioactive compounds in LAB metabolic activities. Benzoic acid, though inhibitory at elevated concentrations, was present at LAB-tolerable levels, maintaining medium stability without negatively impacting growth. Gallic acid functions as a protective agent, enhancing bacterial resilience under oxidative stress conditions and promoting survival during metabolism. Similarly, quercetin aids bacterial survival in oxidative environments, indirectly supporting growth. Trans-cinnamic acid stimulates adaptive metabolic responses at low concentrations, potentially improving LAB metabolic flexibility, although careful control of concentration is necessary to prevent inhibition.

These findings underscore the critical role of phenolic acids and flavonoids in optimized plant-based media formulations. While the significant presence of these compounds contrasts with reports of their inhibitory effects on specific LAB strains, the data indicate that controlled concentrations and compositions in SBB media can beneficially modulate LAB growth and metabolic activity.

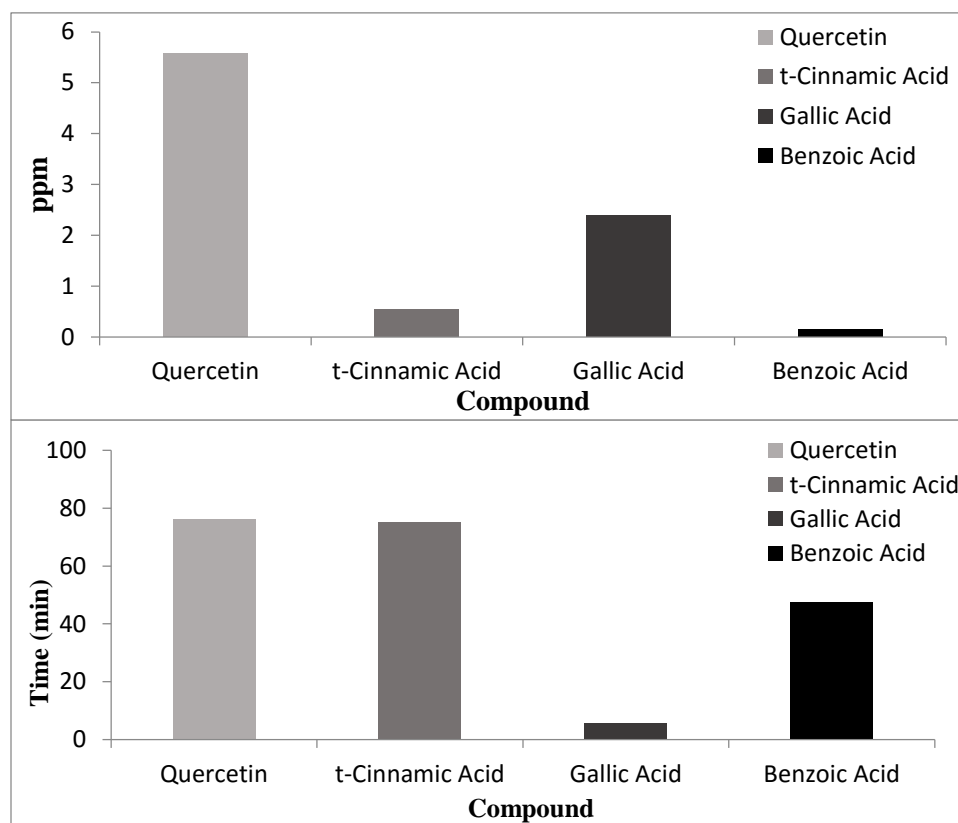


Figure 50. Compounds present in the SBB medium: (a) retention time; (b) quantities.

3.6. High-Performance Liquid Chromatography

Figure 6 presents the growth parameters of selected LAB strains cultivated in MRS and SBB media, highlighting the correlation between key bioactive compounds and their concentrations. Calibration curves for benzoic acid, gallic acid, quercetin, and trans-cinnamic acid showed strong correlations (r^2 values ranging from 0.99962 to 0.99998), confirming accurate quantification of these compounds. Figure 7 displays the HPLC chromatogram, revealing four significant peaks corresponding to these compounds. Quercetin exhibited the highest concentration (47.629 ppm) with a retention time of 47.6 minutes, indicating its predominant role in LAB growth regulation. Gallic acid and benzoic acid were present in lower concentrations (5.912 ppm and 76.313 ppm, respectively), yet still demonstrated strong correlations, suggesting their involvement in modulating LAB metabolism. Trans-cinnamic acid, detected at 7.207 ppm, aligns with its recognized influence on bacterial growth.

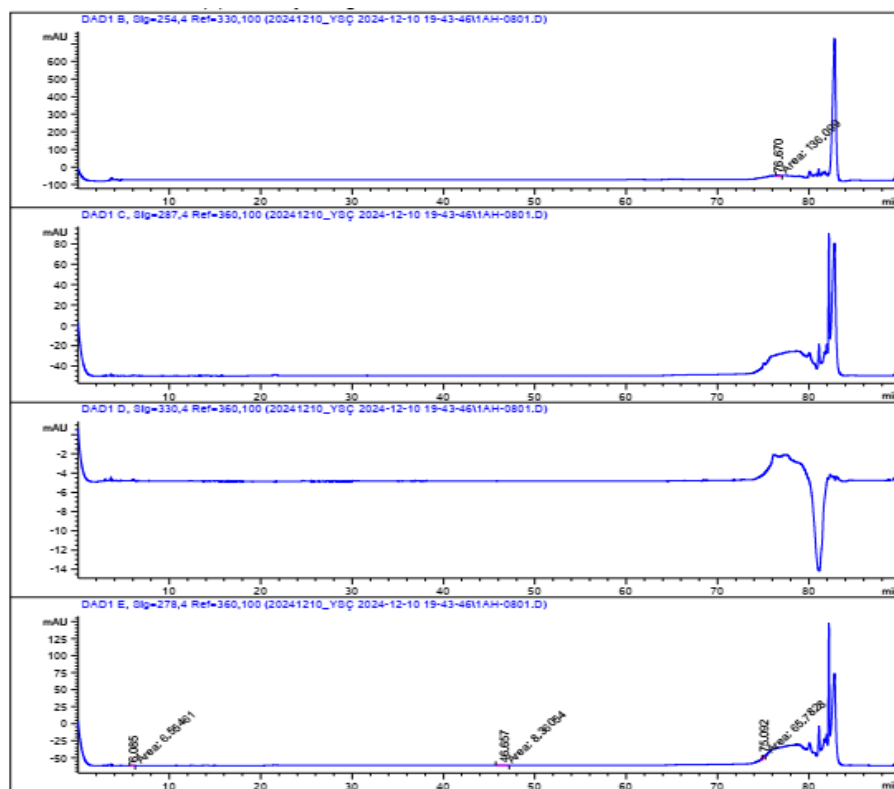


Figure 51. The HPLC chromatograms of the medium soybean beetroot blend (SBB) medium, where: (1) Quercetin; (2) t-Cinnamic Acid; (3) Gallic Acid; (4) Benzoic Acid.

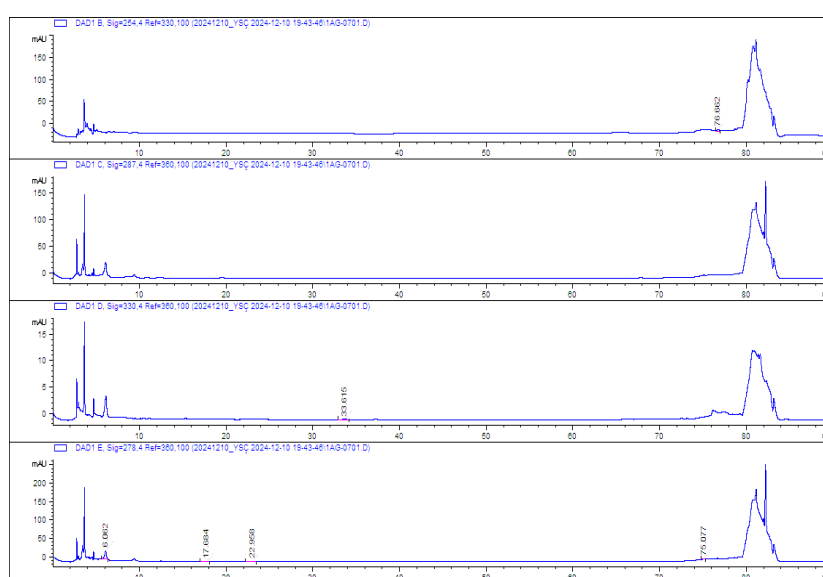



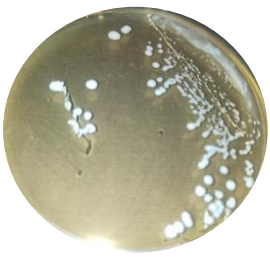


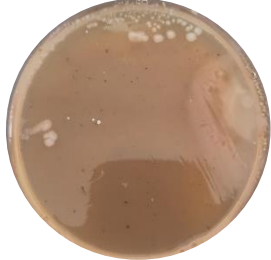

Figure 52. The HPLC chromatograms of the medium soybean beetroot blend (CBB) medium

3.7. Agar plate growth

The agar plate culture of the lactic acid bacteria displayed clearly distinguishable colonies, evenly distributed and easily enumerable. Consistent colony morphology and uniform sizes indicate bacterial purity and optimal growth conditions. The colonies were mostly white or milky white, with a round shape, neat edges, and a moist, smooth surface.

Although MRS medium consistently supports the best performance across all LAB strains, our findings demonstrate that our plant-based media, composed of beetroot and soybean, especially when supplemented with appropriate additives, have the potential to substitute or supplement conventional media like MRS.^{18, 44, 40} Further optimization of these plant-based formulations could lead to more sustainable, cost-effective, and eco-friendly alternatives for LAB cultivation in various biotechnological applications.^{41, 43} These results underscore the clear advantages of MRS medium for acid production and productivity. However, the SBB medium was found to be the most favorable for bacterial growth, followed by BJ-T80, demonstrating that soybean extract could replace Tween 80 while still enhancing LAB growth. This suggests that with further optimization, plant-based formulations could serve as cost-effective and sustainable alternatives to MRS for LAB growth

Table 17. Growth result of the formulated medium, which showed lactic acid bacterial growth.

Medium	Cultural aspect		
	<i>L.plantarum</i>	<i>E. durans</i>	<i>L. Fermentum</i>
MRS ¹			
BJ-T80 ²			

SBB³





¹MRS: Man, Rogosa and Sharpe; ²BJ-T80: Beetroot juice auditioned with Tween 80; ³SBB: Soybean beetroot blend

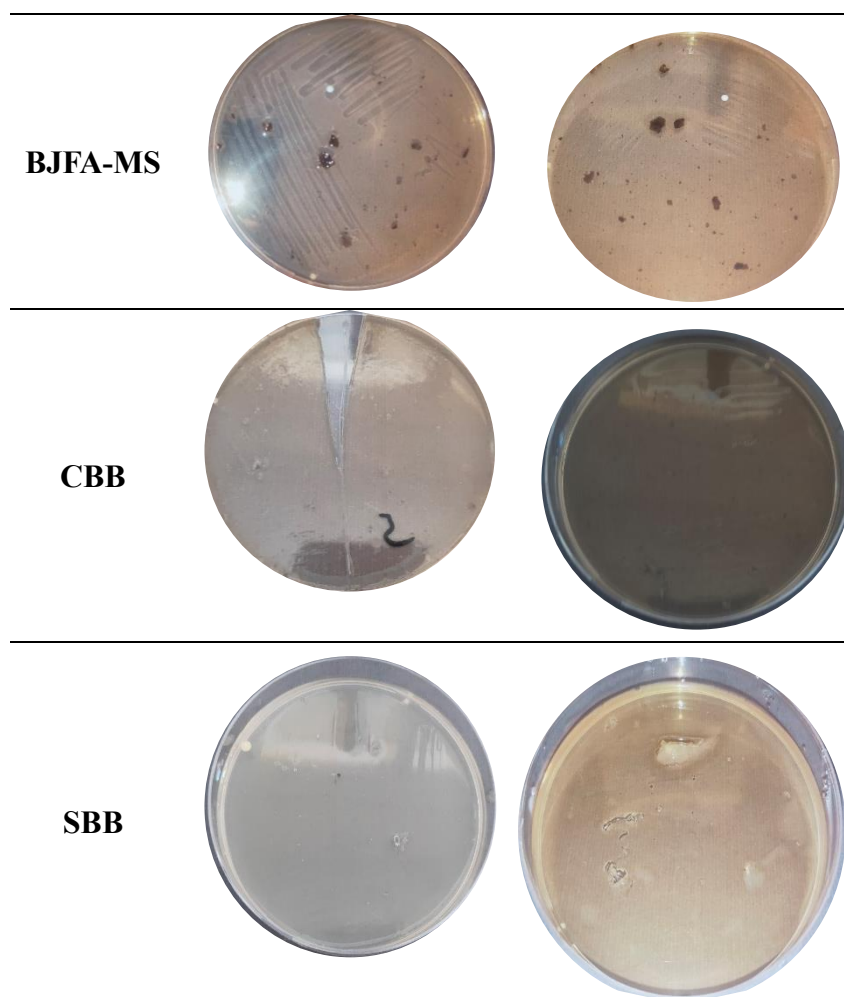
3.8. Results of the selectivity test

Table 18. Results of the selectivity test for the chosen medium

	24 h		72h	
	<i>S. Aureus</i>	<i>E. coli</i>	<i>S. Aureus</i>	<i>E. coli</i>
MRS	-	-	-	-
BJFA-MS	-	-	-	-
CBB	-	-	-	-
SBB	-	-	-	-

Table 19. Aspects of the selectivity teste on the following medium ; BJFA-MS: Beetroot Fixd Additive Manganese Sulfate, CBB: Carob Beetroot Blend SBB: Soybean Beetroot Blend

	72 h	
	<i>S. Aureus</i>	<i>E. coli</i>
MRS		



The selectivity of Man, Sharpe, and de Rogosa (MRS) medium is principally ascribed to the controlled chemical composition of ammonium citrate, as well as magnesium sulfate, which regulates metal ions availability and enzyme activities (Li et al., 2019). Ammonium citrate functions as a chelating agent and metabolic substrate, and it complexes divalent cations (Fe^{2+} , Ca^{2+} , and Mg^{2+}) and hence limits the availability of these ions to bacteria that rely on these ions for membrane stability and enzyme function, like *Staphylococcus aureus* and *Escherichia coli*. However, some homolactic fermenting bacteria, such as lactic acid bacteria (LAB) broadly utilizing these ions efficiently via well-described transport systems, have a physiological advantage due to their ability to also metabolize citrate through the citrate-oxaloacetate-pyruvate pathway. Magnesium sulfate provides key cofactors for LAB enzymatic reactions but keep concentrations below an optimal level for many other bacteria. Together with sodium acetate and the low intrinsic pH of the medium, these components establish an environment that promotes acidophilic, fermentative organisms but inhibits neutrophilic pathogens.

In contrast, CBB, SBB, and BJFAMS show similar selective effects via natural and functional analogues of these compounds. Selectivity from CBB can be originates from polyphenols and

tannins from carob and beetroot, which cause oxidative stress and inhibit cell wall synthesis in *S. aureus* and *E. coli* respectively but the organic acids and sugars from the beetroot promote fermentative growth. The wealth of abundant amino acids and isoflavones from soybeans helps to promote LAB metabolic capacity, and further suppress pathogens by mild phenolic toxicity and acid stress (SBB). BJFAMS, containing manganese sulfate, increases oxidative stress tolerance of LAB, as manganese-dependent enzymes including superoxide dismutase are upregulated, while it causes metal homeostasis disruption in *E. coli* and *S. aureus* that depend on more stringent Mg^{2+} and Fe^{2+} homeostasis.

Thus, the selective characteristic of these media emerges from the synergistic action of physicochemical constraints and metabolic benefits. Chelating agents, scarcity of divalent cations, organic acids, and phenolic compounds form an ecological niche favourable to acid-tolerant, microaerophilic fermenters while inflicting ionic stress, oxidative imbalance, and membrane destabilization in non-target bacteria. As a result, the classical MRS formulation and the beetroot-based media present selective antimicrobial activity on luminal *Staphylococcus aureus* and *Escherichia coli*, showing how the ion balance and acidity, as well as natural antimicrobials, can be used to promote the establishment of beneficial microbiota over pathogens.

Table 20. Summary Results of kinetics fermentation of this thesis different formulation with the lactic acid bacteria

Fermentations Media	μ_{max} (h^{-1})						Generation time G (h)				
	<i>L.</i>		<i>E.</i>		<i>L.</i>		<i>L.</i>		<i>E.</i>	<i>L.</i>	
	<i>plantarum</i>	<i>durans</i>	<i>plantarum</i>	<i>durans</i>	<i>fermentum</i>	<i>plantarum</i>	<i>durans</i>	<i>fermentum</i>	<i>plantarum</i>	<i>durans</i>	<i>fermentum</i>
MRS ¹	0.0138	0.0276	0.0242	0.0242	50.22	25.11	28.64				
Beetroot alone	0.030	0.091	0.044	0.044	23.10	07.61	15.75				
BJFA ²	0.035	0.060	0.039	0.039	19.80	11.55	17.77				
BJFA-MS ³	0.015	0.073	0.036	0.036	46.21	9.49	19.25				
BJFA-MG ⁴	0.014	0.112	0.031	0.031	49.51	6.19	22.36				
BJFA-AS ⁵	0.032	0.077	0.049	0.049	21.66	9.00	14.14				
BJFA-ZO ⁶	0.010	0.109	0.009	0.009	69.31	6.36	77.01				
BJFA-CA ⁷	0.043	0.081	0.022	0.022	16.12	8.56	31.50				

BJFA-PP ⁸	0.032	0.031	0.025	21.66	22.36	27.72
CA ⁹	0.139	0.115	0.131	4.978	6.017	5.303
BJP ¹⁰	0.061	0.097	0.137	11.35	7.163	5.076
CBB ¹¹	0.251	0.19	0.275	2.76	3.639	2.524
SA ¹²	0.316	0.336	0.327	0.030	0.025	0.027
BJ-T80 ¹³	0.262	0.536	0.430	0.043	0.061	0.062
SBB5 ¹⁴	0.161	0.701	0.530	0.028	0.029	0.028

1: MRS: Man de Rogosa Sharp, 2: Beetroot Juice Fixed Additives; 3: SM: sulphate manganese, 4: sulphate Magnesium Sulfate, 5: Acetate Sodium, 6: Zinc oxide, 7: Citrate Ammonium, 8: phosphate dipotassium; 9: Carrob Alone; 10: Beetroot juice peptone; 11: Carroub Beetroot Blend ; 12: Soybean Alone; 13: Beetroot Juice and Tween 80; 14: Soybean Beetroot Blend

Table 21. Summery Results of kinetics fermentation of this thesis different formulation with the lactic acid bacteria

Fermentations Media	Q acid(h-1)			Productivity (h)		
	<i>L. plantarum</i>	<i>E. durans</i>	<i>L. fermentum</i>	<i>L. plantarum</i>	<i>E. durans</i>	<i>L. fermentum</i>
MRS 1	1.588	4.055	1.852	2.1	2.531	2.1
Beetroot alone	0.31	0.29	0.32	0.155	0.14	0.16
BJFA-MS2	1.845	3.819	4.051	1.7	1.867	1.8
CA3	0.296	0.573	0.378	0.034	0.025	1.282
BJP4	0.305	0.434	0.424	0.062	0.031	0.062
CBB5	0.211	0.321	0.378	1.129	1.355	1.282
SA6	0.316	0.336	0.327	0.03	0.025	0.027
BJ-T807	0.262	0.536	0.43	0.043	0.061	0.062
SBB8	0.161	0.701	0.53	0.028	0.029	0.028

1: MRS: Man de Rogosa Sharp, 2: Beetroot Juice Fixed Additives sulphate manganese, 3: Carob Alone; 4: Beetroot juice peptone; 5: Carroub Beetroot Blend ; 6: Soybean Alone; 7: Beetroot Juice and Tween 80; 8: Soybean Beetroot Blend

General discussion

Our choices of plants were in an endeavor toward making a holistic and complete medium that utilized the best of the plant and satisfied the trophic profile of the lactic acid bacteria and the base of our choice is illustrated in table 20

Table 22. Justification of Choosing Criteria for the plants

Criteria	Beetroot	Carob	Soybean
Nutritional Content	High in sugars (sucrose, glucose), betalains, phenolic acids	Rich in polysaccharides, sugars, flavonoids, tannins	Good source of protein, essential amino acids, oligosaccharides
Main usage role	As source of Sugar	as a source of Polysaccharide	Proteinic and amino acid base + Replacement of T80
Availability	Locally grown in Mascara	Abundantly available in Algeria	Currently several Areas Cultivated it in Algeria
Economic Viability	Cost-effective and widely available	Economically viable with underutilized potential	Cost-efficient with extensive agricultural support

First, Beetroot juice alone was tested and successfully supported LAB growth but only to a limited degree in the fermentation period, due to its insufficient nutritional profile and its inadequacy as a stand-alone medium for the tropically demanding LAB. Significantly higher growth was observed when beetroot juice was supplemented with add-in namely : meat extract, yeast extract, peptone, and Tween 80, indicating that nutrient enrichment markedly enhances bacterial development, thus, opening the path of modifying and enhancing the base of beetroot juice by other add-ins. The add-ins were chosen based on reverse engineering from the one proven in MRS medium (satisfying the requirement of suitability and selectivity) and all have a function in the medium (Table 21) . Magnesium sulfate, Ammonium citrate and di-potassium phosphate further improved growth,

though still at levels below those obtained with the standard MRS medium. Notably, the BJFA-MS (Manganese Sulfate) formulation achieved growth patterns nearing the MRS.

Table 23. Key Selective Components used in Beetroot formulation medium.

Component	Role in Selectivity	Mechanistic Explanation
Ammonium citrate	Chelating and metabolic selectivity	Citrate complexes with metal ions (especially Fe^{2+} , Ca^{2+} , Mg^{2+}) and limits their availability.
		Gram-negative bacteria such as <i>E. coli</i> depend more on freely available divalent cations for membrane stabilization (especially Mg^{2+} and Ca^{2+}).
		Lactic acid bacteria tolerate lower free cation concentrations due to their adapted transport systems.
		Citrate also supports lactobacilli metabolism through the citrate fermentation pathway (citrates \rightarrow oxaloacetate \rightarrow pyruvate), providing growth advantage.
Magnesium sulfate	Co-factor selectivity	Mg^{2+} is an essential cofactor for many enzymes, including DNA polymerases and kinases.
		Lactic acid bacteria have efficient Mg uptake and utilization systems; suboptimal Mg^{2+} levels in MRS are balanced for them but often below what <i>Staphylococcus aureus</i> or <i>E. coli</i> require for optimal growth.
Sodium acetate	Inhibitory compound	Inhibits many Gram-negative bacteria and some Gram-positives by diffusing through membranes and acidifying cytoplasm.
		Lactic acid bacteria are relatively tolerant due to active acetate metabolism.
Low pH (≈ 5.5–6.0)	Environmental selectivity	Acidic pH suppresses <i>E. coli</i> and <i>S. aureus</i> but allows <i>acidophilic lactobacilli</i> .

Second, we moved further in the path to enhance the base medium of beetroot juice by utilizing another powerhouse plant rich with nutriment in an attempt to compensate the yeast extract and peptone supplementation to a certain degree or even replace them definitely. Growth performance and productivity analyses revealed that CBB (Carob Beetroot Blend) exhibited the high growth rates for *L. plantarum* (0.251 h^{-1}) and *L. fermentum* (0.275 h^{-1}), surpassing the one of MRS medium, making CBB as an effective medium for LAB cultivation. this result contrasted to the

one of BJFA-MS with The generation, specific acid production rates and productivity were scored better in CBB, in contrast to MRS And BJFA-MS highlighting its potential.

this indicates that Carob addition enhances the formulation from Beetroot and made LAB metabolism function better (Liu et al., 2016). This combination promoted superior growth and production, reducing the need for peptone supplementation.

Third, we moved further in the path to enhance the base medium after the positive results by the Carob addition and the replacement of Peptone usage. We moved further to replacing other critical component which are Meat extract and Tween 80 by utilizing another plant rich in various nutriments. Soybean (*Glycine max*) is a highly nutritious legume with more than nine amino acids recorded in its composition and contain phytosterols and lecithin which has the emulsifying aspect similar to Tween 80 (Lederberg, 1965). Soybean Extract contains a complex mixture of proteins, lipids (including linoleic acid and oleic acid), carbohydrates, and other bioactive compounds(Ayu et al., 2023). The lipids in soybean extract, especially linoleic acid, are valuable fatty acids that LAB may use for growth, On the other hand, Tween 80 is designed to emulsify hydrophobic compounds, facilitating LAB's access to oils like oleic acid, which is more readily bioavailable in Tween 80 compared to simpler fatty acids such as linoleic acid in soybean extract (Reitermayer et al., 2018)(Vignolo et al., 1995). refere to Table 22. Our study demonstrates that our formulation namely Soybean-Beetroot blend (SBB) supports Lactic acid bacteria growth in a comparable to the MRS medium. Although *L. plantarum* exhibited robust growth in this formulation, overall acid production and productivity remained inferior to MRS. This discrepancy underscores the necessity for further optimization of the soybean-beetroot medium to enhance LAB proliferation and acid production effectively.

Table 24. Comparison in amino acid profile of Soybean and Meat extract

Amino Acid	Soybean Extract	Meat Extract
Alanine	Present	Present
Arginine	Present	Present
Aspartic Acid	Present	Present
Cysteine	Present	Present
Glutamic Acid	Present	Present
Glutamine	Present	Present
Glycine	Present	Present
Histidine	Present	Present
Isoleucine	Present	Present
Leucine	Present	Present
Lysine	Present	Present
Methionine	Present	Present
Phenylalanine	Present	Present
Proline	Present	Present
Serine	Present	Present
Threonine	Present	Present

Tryptophan	Present	Present
Tyrosine	Present	Present
Valine	Present	Present
B12-dependent amino acids	Absent	Present
	(Boateng et al., 2023)	(Ramin Jorfi, 2012)

This table provides a simplified comparison of amino acids present in both Soybean and Meat Extract, they are similar in the majority and provide full amino acid needed by LAB growth. The notable disarray is that B12-dependent amino acids (those associated with cobalamin) are absent in soybean extract but present in meat extract. This component is required for the growth of fastidious LAB, especially those that rely on medium bioavailable B12 for coenzyme synthesis and other metabolic functions.

Fourth, pigments such as carotenoids and betalains are mentioned to be sensitive to low pH values. However, betalains show higher stability at low pH compared to carotenoids (Jackson, 2020; Marszałek et al., 2021; Sajjad et al., 2020) This could be due to the hydrolyzation process where glucose is obtained from betanin, which is a glycoside, and glucose and betanidin are created and since these LAB strains can use this glucose and betanidin as a sugar source (Esteves et al., 2018)

Regarding the discoloration of the medium, Lactic acid bacteria (LAB) has the ability to degrade betalain pigments, such as betacyanins and betaxanthins, present in beetroot through specific enzymatic activities. The key enzymes involved in the degradation of betalains by LAB include:

1. **Betanase (Betacyanin dehydrogenase):** This enzyme catalyzes the breakdown of betacyanins (the red pigments in beetroot) converting it into non-pigmented products such as betalamic acid. Betanase has not been fully identified in LAB strains(Wang et al., 2019).
2. **Polyphenol oxidases (PPO):** certain LAB strains namely *L. plantarum* possess polyphenol oxidases, which can oxidize phenolic compounds and might assist in the breakdown of betalain pigments through a more indirect pathway (Łepecka et al., 2024).
3. **β -glucosidase:** This enzyme hydrolyzes the glycosidic bonds in the betalain molecules, breaking them down into simpler forms, although its role in betalain degradation is less pronounced than betanase (Nemet et al., 2017).

3.8.1. Contrasting with other research related to MRS and modified version

Numerous modified MRS (de Man, Rogosa and Sharpe) media have been developed to meet specific growth and selectivity needs of lactic acid bacteria (LAB).

- **Low-cost modified MRS (mMRS):** This formulation has a simplified composition containing glucose, yeast extract, dipotassium phosphate, manganese sulfate monohydrate, Tween 80, and anhydrous sodium acetate. It is designed to increase bacteriocin production efficiency while reducing medium cost.
- **Tomato based medium:** Yoon KY et al. (2004) demonstrated tomato juice can serve as a raw material for probiotic juice production by several LAB strains (*L. acidophilus*, *L. plantarum*, *L. casei*, and *L. delbrueckii*). Fermentation lowered pH and achieved viable cell counts up to 10^9 CFU/ml.
 - we cite the works of Abdel-Malek *et al* 2007 has been involved in research related to lactic acid bacteria (LAB) and tomato. One relevant study reports the isolation and evaluation of LAB strains fermented in a tomato based medium.
- **Soytone medium:** A plant-based medium derived from soy protein components designed to replace animal-derived peptones and extracts in classic MRS for cost-effective high-density cultivation of Lactobacillaceae. It optimizes nitrogen and carbon sources from plants to maximize biomass .(Jona et al., 2025) . Positive growth was reported on this medium for , *L. crispatus* *L. crispatus*. The latter was 45% higher than when cultivated in MRS Broth (Jona et al., 2025).

These formulations exemplify common strategies for adjusting MRS components to optimize LAB growth, enhance selectivity, and improve metabolite production, with exact compositions depending on experimental objectives and target organisms. While our approach aims to provide a medium that similar in potential and different in composition with ;ore available and affordable alternatives.

Conclusion
Limitation and perspectives

Conclusion

This study provides compelling evidence for the viability of plant-based media as a potential alternative for the traditional Man de Sharp Rogosa medium an in-depth contrasting between the proposed medium and the MRS, through fermentation by pH, acidity and Optical density follow-up and the monitoring of the Kinetic parameters, namely: the maximum specific growth rate (μ_{max}), generation time (G), acid production rate (Q_{ac}) and productivity (g/L/h). Through a comprehensive examination of beetroot-based media (BJFA-MS), including formulations enhanced with Carob (CBB), Soybean (SBB), we have demonstrated significant advancements in microbial growth and metabolic activity. These formulations namely BJFA-MS, CBB and SBB showing comparable or in certain cases superior growth rates to MRS, particularly for strains such as *Lactobacillus plantarum*, *Lactobacillus fermentum* and *Enterococcus durans*.

While beetroot juice alone showed limitations due to its inadequate nutritional profile, the incorporation of essential nutrients and minerals like manganese sulfate in BJFA-MS significantly enhanced bacterial growth. Moreover, the addition of Carob and soybean in separate manner to beetroot formulation and contrast the result with the stand alone medium led to showcasing the further improvements results, with the CBB medium outperforming MRS in terms of growth rates and acid production. While The SBB medium also exhibited strong performance, particularly for *L. plantarum*. The HPLC analysis of phenolic compounds in CBB, SBB mediums indicated that benzoic acid, gallic acid, quercetin, and trans-cinnamic acid presence could have significantly influenced LAB growth, further validating the beneficial properties of these compounds in optimizing microbial media.

In conclusion, the study underscores the potential of plant-based media, particularly beetroot-derived formulations as eco-friendly, nutritionally balanced and efficient alternatives to MRS. These findings not only contribute to the development of sustainable and environmentally friendly microbiological media but also pave the way for future research into plant-based substrates in biotechnological applications. By offering a practical solution for cost-effective and sustainable LAB cultivation, this research highlights the promise of integrating natural resources into modern fermentation processes, with significant implications for food biotechnology and microbial research.

Usage indication

This medium offers a cost-effective alternative to the MRS medium for the repropagation and general inoculation of lactic acid bacteria, supported by its favorable interaction with bacterial components. It provides optimal growth conditions without the extensive use of MRS, making it suitable for routine inoculation and microbial culture propagation. While the current state of knowledge highlights its effectiveness, ongoing studies on further characterization and enhanced selectivity may reveal additional perspectives and applications, potentially refine its role and increase its specificity for various bacterial strains.

Limitations of the Study

While this study provides promising insights into the potential of plant-based media, particularly beetroot-derived formulations as alternatives for Lactic Acid Bacteria (LAB) cultivation, several limitations must be addressed in future research :

1. **Nutrient Variability:** The quality and nutrient composition of plant-based materials such as beetroot, carob, and soybean can vary based on geographic location, cultivation methods, and environmental factors. This variability may impact the reproducibility and consistency of results across different batches or regions, limiting the scalability of the proposed media for industrial use.
2. **Optimization for Specific Strains:** While formulations like BJFA-MS and CBB showed promise, some LAB strains, especially *Lactobacillus plantarum* and *Lactobacillus fermentum*, exhibited differences in their growth kinetics across the media. This variability suggests that further optimization is needed to tailor the media for specific strains and improve performance for industrial fermentation requirements.
3. **Lack of Long-Term Stability Testing:** The study did not explore the long-term stability of beetroot-based media in industrial-scale fermentation. Understanding the shelf-life and storage conditions of these plant-derived media is crucial for their practical applications in the food and biotechnology industries.
4. **Laboratory-Scale Limitations:** The experiments were conducted on a small scale under controlled laboratory conditions. The scalability and industrial reproducibility of the medium formulations remain untested, which may present challenges in real-world applications where process conditions vary significantly. Further studies should focus on large-scale fermentation trials to evaluate the performance of these formulations in more dynamic industrial environments.

Perspectives for Future Research

Despite the aforementioned limitations, this study opens several avenues for future research to further enhance the sustainability and efficiency of plant-based media for LAB cultivation:

1. **Comprehensive Nutrient Profiling:** Future studies could focus on identifying the specific nutrients required for optimal LAB growth in beetroot-based media. Advanced nutrient

profiling and the addition of targeted supplements could help refine formulations and improve their performance across different LAB strains.

2. **Formulation Optimization:** To address the limitations of acid production, future research could focus on optimizing beetroot-based formulations by exploring additional plant-based additives, such as prebiotics or oligosaccharides, to boost LAB metabolism and enhance acid production efficiency.
3. **Microbial Strain-Specific Media Development:** Given that different LAB strains exhibited varying growth performances, further studies could explore strain-specific medium optimization. This would involve tailoring media to the particular nutritional requirements of each strain, thus improving overall microbial growth and productivity.
4. **Large-Scale and Industrial Testing:** Future work should focus on scaling up the promising formulations from laboratory conditions to larger industrial fermentation systems. This would involve assessing the performance of beetroot-based media in bioreactors, evaluating long-term stability, and determining the feasibility of these media for large-scale production.
5. **Environmental and Economic Impact:** A broader perspective on the environmental and economic impacts of switching to plant-based media is necessary. Future research could quantify the cost savings, energy efficiency, and environmental benefits of using beetroot-derived media compared to traditional synthetic alternatives like MRS.
6. **Exploring Other Plant-Based Substrates:** While beetroot has shown promise, exploring a wider range of plant-based substrates (such as other root vegetables or fruit juices) in combination with beetroot could lead to new, more effective, and environmentally friendly alternatives for LAB cultivation. Testing these alternatives across various LAB strains could further diversify and optimize microbial cultivation media.

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Article 1.

Formulations of novel microbial medium from beetroot powder: a sustainable substitute for commercial Man-de-Rogosa Sharp agar (MRS) in cultivating lactic acid bacteria.



Formulations of novel microbial medium from beetroot powder: a sustainable substitute for commercial Man-de-Rogosa Sharp agar (MRS) in cultivating lactic acid bacteria

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Abstract

This preliminary study investigates the feasibility of utilizing Algerian beetroot as a sustainable alternative to commercial Man-de-Rogosa Sharp Agar (MRS) for cultivating lactic acid bacteria. The research explores the optimization of microbial media formulations incorporating beetroot powder and assesses its efficacy in supporting the growth of *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Enterococcus durans*. Methodologically, vegetal samples were harvested and dried, bacterial strains prepared, and phytochemical analyses conducted. Titratable acidity and sugar content in the beet-based medium were measured, revealing pH 5.5, 13% titratable acidity, and 67 g/L sugar content. Results indicate that while beetroot juice alone exhibited limited bacterial growth due to its inadequate nutritional properties, formulations incorporating additives such as magnesium sulfate, ammonium sulfate, and di-potassium phosphate showed improved growth. Particularly, the formulation "BJFA-SM" (with manganese sulfate) demonstrated comparable growth to MRS, with reduced generation times for *L. fermentum* and *E. durans*. Additionally, the disappearance of betalain pigment from beetroot-based media indicates successful bacterial growth. Agar plate testing has revealed similar macroscopic characteristics and cell counts to the MRS medium indicating the potential of beetroot-based media as a sustainable substitute for traditional growth media. Finally, the BJFA-MS medium showed superior growth for *L. plantarum*, *E. durans*, and *L. fermentum* compared to MRS, with faster growth rates. However, it exhibited lower acid production efficiency than MRS. This study underscores the viability of beetroot-derived formulations as eco-friendly alternatives for cultivating lactic acid bacteria, with implications for sustainable microbiology research and biotechnological applications.

Keywords *Lactobacillus plantarum* · Natural media · Bacterial growth · Beetroot juice

Introduction

The complex relationship between the environment and human health has gained considerable attention in recent years, leading to a transition towards sustainable practices and the exploration of natural alternatives across various fields. An essential facet within this framework involves the cultivation of lactic acid bacteria (LAB), a pivotal contributor to processes such as food fermentation, probiotics, and the promotion of health. Notably, the *Lactobacillus* species within the LAB family have emerged as key contributors due to their diverse applications and potential health advantages. The selection of growth media is central to the cultivation of these strains, as it influences critical factors such as growth rate, metabolism, and viability. In light of this context, the present study delves into the growing interest

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in sustainable alternatives to synthetic LAB growth media, driven by environmental concerns and an increasing preference for microbiota-rich foods (Mokoena 2017). A compelling option in this realm is the utilization of beetroot (*Beta vulgaris*) as a growth medium. Beetroot is widely recognized for its nutritional richness and abundance of bioactive compounds, making it a promising natural and sustainable substrate for promoting *Lactobacillus* growth. Our research aims to uncover the untapped potential of red beetroot as a carrier for probiotics and a natural growth medium for *Lactobacillus* strains. By leveraging the nutritional components inherent in beetroot, such as sugars, vitamins, and phytochemicals, our formulation anticipates enhancing the growth and metabolic activities of *Lactobacillus*. The study encompasses a comprehensive assessment, ranging from the selection of *Lactobacillus* strains and the preparation of beetroot medium to cultivation experiments and the evaluation of growth kinetics and lactic acid production (Wang et al. 2021). Through this exploration, we aim to contribute to the expanding field of natural-based bioprocessing by providing insights into sustainable strategies for improving LAB cultivation. The overarching goal is to lay the groundwork for the advancement of inventive functional foods, probiotics, and environmentally sustainable microbial supplements. In doing so, our work aligns with the broader shift towards environmentally conscious practices and the pursuit of healthier, sustainable alternatives (Souza et al. 2005).

Materials and methods

Harvesting and drying procedure of vegetal samples

The beet pulps were provided from a local farm in the north-west region of Mascara, Algeria. The beets were meticulously cleaned allowing removal of any residual soil, and the leafy portion was selectively trimmed to retain solely the root section for preservation. Afterward, segments of beetroot pulp are peeled and ground then left to dehydrate naturally in atmospheric temperature. The dehydrated grind beetroot is then turned into powder and stocked.

Bacterial strains and culture conditions

Three Lactic acid bacteria strains, namely: *Lactobacillus plantarum* (JCM1149), *Lactobacillus fermentum* (NBRC15885), and *Enterococcus durans* (JCM8725) were selected for this study (Oussama et al. 2023). The bacterial strains were obtained from a reputable culture collection and were stored at $-80\text{ }^{\circ}\text{C}$ in glycerol stocks. For cultivation purposes, the strains underwent two successive

propagations in a suitable growth medium (MRS broth) at $37\text{ }^{\circ}\text{C}$ for 24 hours.

Phytochemicals analyses In this study, 100 g of dried beetroot powder was finely ground to a uniform particle size. The sample was then subjected to conventional laboratory analyses to determine its nutritional composition. Calibration standards for each nutritional component—total sugar, protein, moisture content, fat percentage, fiber content, and humidity—were prepared according to established protocols. These standards were analyzed using relevant analytical techniques, including but not limited to spectrophotometric methods, Kjeldahl nitrogen determination, Soxhlet extraction for fat content, and gravimetric methods for moisture and fiber analysis. For moisture content determination, a drying oven method was used, with the sample placed in a pre-weighed container and heated at $105\text{ }^{\circ}\text{C}$ until a constant weight was achieved. Total sugar content was assessed using a phenol-sulfuric acid method, while protein content was determined via the Kjeldahl method. Fiber content was analyzed by the detergent method (acid-detergent fiber, neutral-detergent fiber), and fat content was quantified through Soxhlet extraction with petroleum ether. Each analysis was performed in triplicate to ensure the accuracy and repeatability of results (Ingle et al. 2017; Otalora et al. 2020; Shi et al. 2021).

Titrateable acidity

This procedure involves titrating an acidic solution with a known concentration of basic solution to determine titrateable acidity. Titrateable acidity reflects the quantity of acid present in the sample and is expressed in terms of concentration. By adding a base solution (NaOH) to 20 ml of juice containing an acid-base indicator, the solution's color change is observed until it becomes permanent. The residual acidity is determined by measuring the volume of NaOH used, taking into account its concentration, and subsequently multiplying to ascertain the concentration of titrateable acidity in 100 ml of juice.

Total sugar assay procedure

A phenol-sulfuric acid method was employed to quantify total sugars in beet juice. Firstly, a sugar assay reagent solution was prepared following the manufacturer's instructions. Subsequently, 10 ml of beet juice was transferred using a pipette into a 50 ml beaker and then diluted with distilled water to reach a final volume of 50 ml. Using a graduated burette, 1 M sodium hydroxide (NaOH) solution was prepared. The beaker containing the beet juice mixture was heated to boiling using a water bath. Following that,

the sugar assay reagent solution was meticulously added drop by drop to the beaker, with continuous stirring. The resultant-colored compound was quantified to ascertain the total sugar content.

Inoculum Preparation

To reanimate preserved strains, a dedicated culture medium tailored to each strain is prepared. The frozen strain is swiftly thawed by immersing it in a 37 °C water bath until complete thawing is achieved. Subsequently, the thawed strain is transferred to the prepared culture medium using a sterile inoculation loop. The tubes or flasks containing the inoculated strains are then incubated at the recommended temperature for each specific strain (35 °C to 37 °C for lactobacilli, 37 °C to 40 °C for enterococci). During the incubation period, the growth of bacterial colonies is monitored. Once completed, the bacteria are transferred to a gelatinous MRS culture medium to continue with experimental procedures.

Beetroot-formulated media fermentation

In the methodological framework of our research on the conceptualization of a beetroot-based medium as a potential alternative to the MRS medium for cultivating lactic acid bacteria, a systematic series of experiments was conducted. The study progressed through distinct phases, starting with an examination of the standalone effects of beetroot juice. Subsequently, fixed additives, including meat extract, yeast extract, peptone, and threonine, were introduced to assess synergistic effects. Finally, the experimental scope expanded to include the integration of specific chemical agents: Magnesium sulfate, Manganese sulfate, Sodium acetate, Ammonium citrate, di-potassium phosphate, and zinc oxide into the beetroot and additive mix. This comprehensive approach aimed to unravel the intricate dynamics among these components, offering insights into the viability of the medium derived from beetroot to facilitate the growth of lactic acid bacteria. With implications for replacing the conventional MRS medium.

Colorimetric analysis of fermentation chosen medium

The color analysis of the juices made in this study involved the utilization of a LICO600 spectrophotometer to conduct colorimetric analysis, aimed at assessing the growth dynamics of three lactic acid bacteria strains: *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Enterococcus durans*. Cultures were incubated under controlled conditions, and samples were extracted at 0, 12 and 24 h. The colorimeter analysis of the beetroot medium+sulfate manganese

inoculated with bacterial cultures was measured using the LICO600 spectrophotometer, providing a quantitative assessment of growth based on changes in color intensity over the specified time intervals. Collected data, comprising absorbance readings for each bacterial strain at each time point, were subjected to statistical analysis to identify patterns and variations in growth profiles. This methodology ensures a comprehensive understanding of the growth dynamics of the specified lactic acid bacteria strains over the investigated periods.

Selection of BJFA-MS and Fermentation Comparative Analysis

The comparative analysis of fermentation between the BJFA-MS and MRS mediums involved fermenting selected lactic acid bacteria strains in both mediums while monitoring acidity, pH, and optical density throughout the fermentation process. Parameters assessed included μ_{max} (maximal growth specific speed), G (generation time), and Qac (acid production speed), and productivity (g/L.h), representing the quantity of acid produced per hour. Fermentation experiments were conducted under controlled conditions, with samples collected at regular intervals for analysis. The obtained data were then subjected to statistical analysis to elucidate differences and similarities between the two mediums in supporting bacterial growth and acid production.

Testing growth on agar plates

To prepare the growth media we start by fermenting substances. Next, we dissolve 4.2 mg of Agar-Agar powder in 250 ml of water. Sterilize it through autoclaving at 121 °C for 15 min. After cooling it down to 42 °C we enhance the solution, with 250 ml of Broth (in our case Beetroot broth) along with fixed additives such as Meat extract Yeast extract, Tween 80, and Peptone. Once the medium is evenly mixed we pour it into Petri plates. Inoculation is conducted by applying an acid suspension of 0.1 ml, possessing a turbidity equivalent to a standardized turbidity level of 1.5×10^8 CFU/ml (corresponding to a turbidity of 0.5 McFarland). We then allow the plates to incubate at a temperature of 37 °C for 24 h. Following and documenting growth, macroscopic appearance, and colony color. To ensure the accuracy, of our findings we also monitor colony viability by reseeded them for analysis.

Statistical analysis and fermentation parameters calculation

The fermentation experiments were conducted in triplicate, and duplicate analyses were performed for each sample. The

results are expressed as the mean \pm SD (standard deviation). Statistical analysis of the experimental data was conducted using the SPSS 13.0 software package. Calculation of fermentation parameters involves determining the kinetic culture parameters, The specific microbial growth rate (μ) can be calculated through the utilization of the semi-logarithmic method to plot $\ln(X/X_0)$ against time as for the time of generation it's expressed as $G = \ln 2 / \mu_{max}$.

Results

Phytochemical analysis

In the results section, we present findings from non-destructive analysis using Near-Infrared Spectroscopy (NEIRS), where meticulous calibration standards were prepared for nutritional components Table 1.

Fermentation

Fermenting beetroot juice with *Lactobacillus plantarum* has been instrumental in facilitating the growth of various lactic acid bacteria. The confirmed presence and diversity of lactic acid bacteria, including *Lactobacillus plantarum*, in beetroot juice indicate its potential for producing probiotic beetroot juice as a non-dairy product. This experiment involved the fermentation of beetroot juice alone and in different formulations by 3 lactic acid bacteria namely *L. plantarum*, *L. fermentum*, *E. durans*. The initial inoculum of all lactic acid bacteria in fermented juices was calibrated to 10^7 log CFU/mL Fig. 1, with optical density serving as an indicator of bacterial growth and medium consumption. Multiple contrasts were observed between the beetroot juice alone and the remaining formulation juice. Note that the medium: Beetroot Juice with Fixed Additives -peptone, meat extract, yeast extract, and tween80 will be referred to as 'BJFA'.

As a start, beetroot juice alone exhibited less growth with all three lactic strains all along the fermentation period, this

can be related to the lack of nutritional properties of beetroot alone and that it's not a stand-alone media for lactic acid bacteria which are known to be exigent in their trophic profiles. A much higher growth was exhibited for the three bacteria when the Beetroot juice was auditioned with: meat extract, yeast extract, peptone, and tween 80, then we noted higher growth during the fermentation compared to the beetroot alone fermentation. The lowest growth after the beetroot juice-based medium was negligible. Notably, the medium containing beetroot auditioned with components such as magnesium sulfate, ammonium sulfate, and di-potassium phosphate exhibited overall improved growth. However, when compared to the MRS medium, bacterial development was less pronounced. A noteworthy formulation that succeeded in this trial was the combination of BJFA -MS" (Beetroot juice Fixed Additives - Manganese Sulfate), achieving comparable results to MRS and following a similar trajectory in bacterial growth. Employing a consortium of symbiotic lactobacilli, including *Lactobacillus plantarum*, proved suitable for obtaining fermented beet juice with elevated organoleptic characteristics and adaptability properties (Machulin et al. 2022).

Moreover, The results of this study are similar to those described by (Malik et al. 2019), They exhibited observable growth in *L. plantarum*, with a progressive increase in viable cell count over time when utilizing beetroot juice (Malik et al. 2019). The study by Wang et al. 2015 realized a similar approach to ours, the method described in their paper involves processing beet juice with *Lactobacillus plantarum* to obtain fermented beet extract. Their results highlight that beetroot contains the mandatory nutrition necessary to develop and grow thus expressing the production of lactic acid and succeeding in fermenting the beetroot medium(Wang, Na., Xiong, Guoxi., Si, Hui., Wang, Ping., Wang 2015).

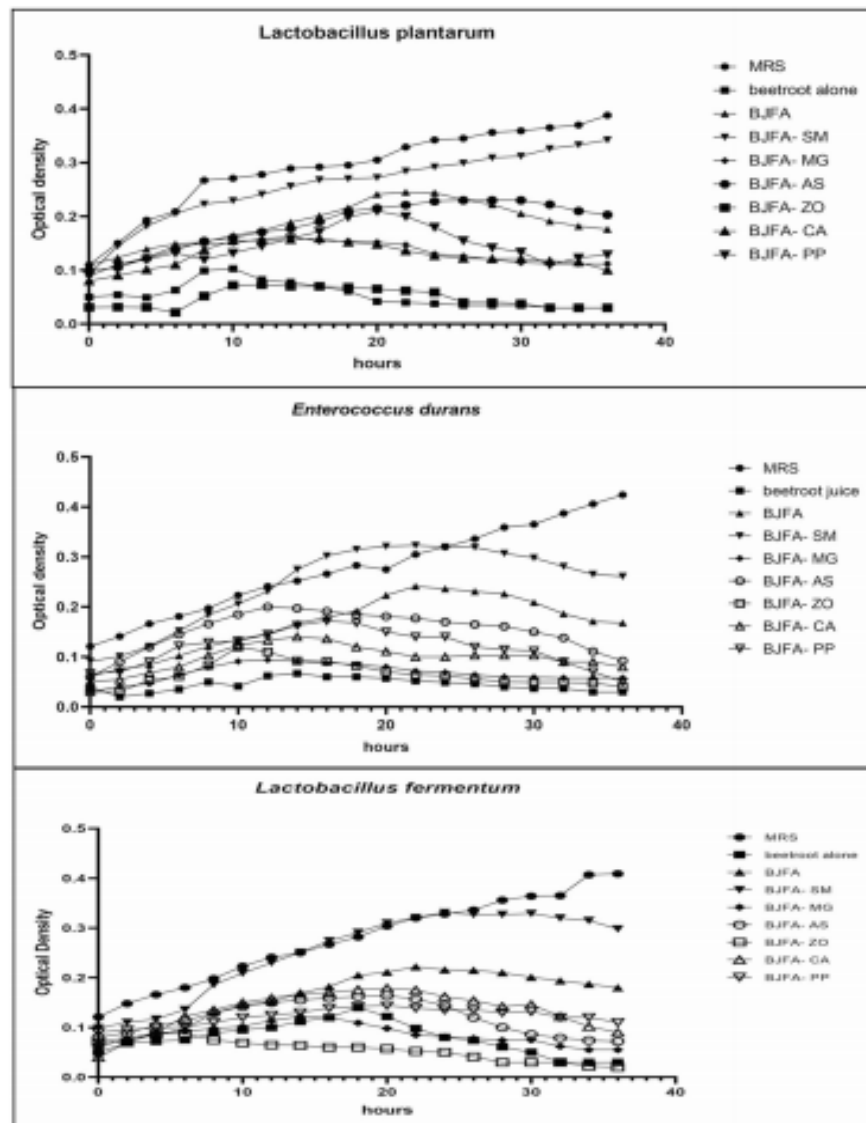
Kinetic parameter of fermentation

Conventionally and since the foundation of Man de Rogosa Sharp known as MRS medium, it is the reference for cultivating and propagating lactic acid bacteria. However, it has become needed to versatile the medium on which we can propagate these beneficial bacteria to further study and utilize them. In this valorization path of our investigation, we verified and crafted a semi-natural medium from beetroot pulp and the minimum nutrition requirement needed for lactic acid bacteria which are very exigent in their trophic profile. The calculation of: the specific speed of Growth μ_{max} (h^{-1}) and the generation time of each medium was performed utilizing data collected from the fermentation. Although we can notice that several formulations from the BJFA exhibit a higher specific Speed of growth it's important to mention

Table 1 Result of phytochemical analysis of the beetroot powder

Parameter	beetroot powder
pH	5.5
Acidity titrable m. eq	13.02
Total sugar	67
le plus abundant	Sucrose
water content/100 g	87.6
Protein	31.09
FAT%	1.32
cellulose(fiber)	18.17
humidity	8.45
Optical density	2.16

Fig. 1 Fermentation on different formulations from BJFA: Beetroot Juice Fixed Additives with *L. plantarum*, *L. fermentum*, and *E. durans*; SM: sulfate manganese, MG: sulfate Magnesium, AS: acetate sodium, ZO: zinc oxide, CA: citrate ammonium, PP: phosphate dipotassium



that the speed of growth has to be coupled with the time of generation to be interpreted properly and compared to the reference media Table 2.

Color-metrics

The color of a juice can be determined precisely by its Yellow*, Red*, and Green* color also referred to as Chlorophyll* values, which respectively represent Yellow, Red/Green proportion (refer to Fig. 2). It is interesting to note that beetroot juice has the highest red value at the start of the fermentation (with an R* value of 1.8–2.0), while beetroot

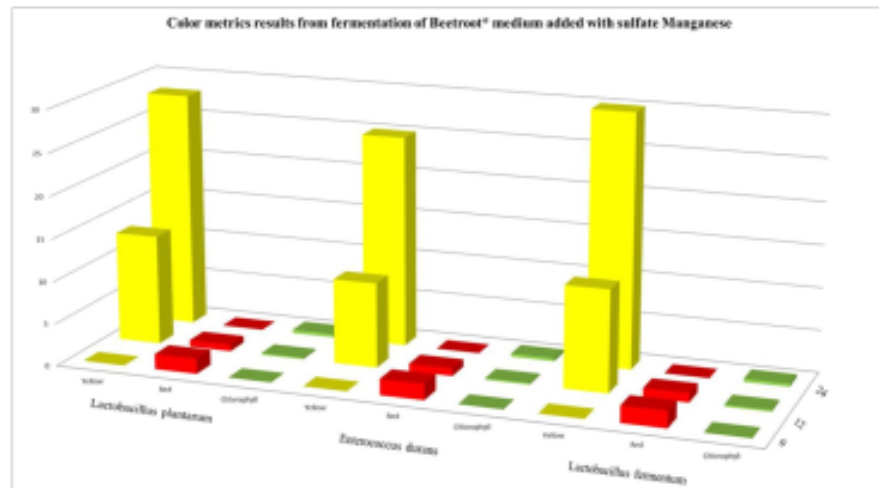
juice's Yellow value at the start is null. As for the Green value at the start, it is negligible and approximately between 0.01 and 0.03. When beetroot juice is fermented with the lactic bacterial strain, the resulting data from the juice display significant changes in the color analysis where the Yellow* value has significantly increased achieving a value between 25 and 30, and the Green*value (chlorophyll) elevated to around 0.37–0.40. The noteworthy change is the disappearance of the Red* value which is due to the presence of betalain pigment typical to beetroot where it was reduced to under 0. This change in the color indexes of the beetroot formulation is probable due to the digestion of

Table 2 Results of kinetics fermentation of different BJFA with the lactic acid bacteria

Fermentations media	μ_{max} (h^{-1})			Temps de génération G (h)		
	<i>L. plantarum</i>	<i>E. durans</i>	<i>L. fermentum</i>	<i>L. plantarum</i>	<i>E. durans</i>	<i>L. fermentum</i>
MRS (reference)	0.0138	0.0276	0.0242	50.22	25.11	28.64
Beetroot alone	0.030	0.091	0.044	23.10	07.61	15.75
BJFA ¹	0.035	0.060	0.039	19.80	11.55	17.77
BJFA- SM	0.015	0.073	0.036	46.21	9.49	19.25
BJFA-MG	0.014	0.112	0.031	49.51	6.19	22.36
BJFA-AS	0.032	0.077	0.049	21.66	9.00	14.14
BJFA-ZO	0.010	0.109	0.009	69.31	6.36	77.01
BJFA-CA	0.043	0.081	0.022	16.12	8.56	31.50
BJFA-PP	0.032	0.031	0.025	21.66	22.36	27.72

¹ Beetroot Juice Fixed Additives, SM sulfate manganese, MG sulfate Magnesium, AS acetate sodium, ZO zinc oxide, CA citrate ammonium, PP phosphate dipotassium

Fig. 2 Graphic representation of the colorimetric results for medium BJFA-MS for *L. plantarum*, *L. fermentum*, and *E. durans*



the nutritional component present in the media due to the increase in the Lactic acid formation and the reduction of pH value. According to the literature, pigments such as carotenoids and betalains are mentioned to be sensitive to low pH values. However, betalains show higher stability at low pH compared to carotenoids (Jackson 2020; Marszalek et al. 2021; Sajjad et al. 2020) This could be due to the hydrolyzation process where glucose is obtained from betanin, which is a glycoside, and glucose and betanidin are created and since these LAB strains can use this glucose and betanidin as a sugar source (Marzo et al. 2021).

Comparative fermentation in BJFA-MS and MRS

The results presented herein offer a comparative analysis of fermentation outcomes between BJFA-(Beetroot Juice Fixed Additive - Manganese Sulfate) and MRS mediums

for three distinct lactic acid bacteria strains. Through an examination of key parameters to elucidate the efficacy of BJFA-MS as a potential substitute for the conventional MRS medium. These findings provide valuable insights into the suitability of BJFA-MS in fostering lactic acid bacteria fermentation and its implications for industrial applications.

Results are presented as the Optical density. In the case of *L. plantarum*, the BJFA-MS medium exhibited a slightly higher maximal growth specific speed (μ_{max}) of 0.015 h^{-1} compared to 0.014 h^{-1} in the MRS medium. Additionally, the generation time (G) was shorter in BJFA-MS (46.2 h) compared to MRS (49.5 h), indicating faster bacterial growth. However, there was a notable difference in acid production speed (Qac) and productivity. While the Qac was higher in BJFA-MS (1.7 g/g.h) than in MRS (1.03 g/g.h), the productivity was lower (1.75 g/L.h in BJFA-MS compared

to 2.1 g/L.h in MRS). Despite this, BJFA-MS demonstrates comparable or slightly enhanced performance in supporting *L. plantarum* fermentation, suggesting its potential as a substitute for MRS medium, especially considering its similar growth parameters and enhanced acid production. As For *E. durans*, the contrast between BJFA-MS and MRS mediums reveals striking differences in bacterial growth parameters. BJFA-MS exhibited a substantially higher maximal growth specific speed (μ_{max}) of 0.074 h⁻¹ compared to 0.028 h⁻¹ in the MRS medium. Moreover, the generation time (G) was significantly shorter in BJFA-MS (9.37 h) compared to MRS (24.57 h), indicating much faster bacterial growth. Additionally, acid production speed (Qac) and productivity were markedly higher in BJFA-MS (3.82 g/g.h and 1.87 g/L.h, respectively) compared to MRS (2.5 g/g.h and 2.53 g/L.h, respectively). These results underscore the superior performance of BJFA-MS in supporting *E. durans*

fermentation, suggesting its potential as a more effective substitute for MRS medium.

Lastly, In the case of *L. fermentum*, BJFA-MS also displayed notable advantages over the MRS medium in several growth parameters. BJFA-MS exhibited a higher maximal growth specific speed (μ_{max}) of 0.05 h⁻¹ compared to 0.034 h⁻¹ in the MRS medium. Additionally, the generation time (G) was considerably shorter in BJFA-MS (13.9 h) compared to MRS (20.39 h), indicating faster bacterial growth. Furthermore, acid production speed (Qac) and productivity were notably higher in BJFA-MS (4.05 g/g.h and 1.8 g/L.h, respectively) compared to MRS (1.85 g/g.h and 2.1 g/L.h, respectively). These findings suggest that BJFA-MS outperforms MRS medium in supporting *L. fermentum* fermentation, highlighting its potential as a more efficient alternative (Fig. 3 and Table 3).

Fig. 3 The growth kinetics of selected *Lactobacillus*, namely, *Lactobacillus plantarum* (a), *Enterococcus durans* (b), *Lactobacillus fermentum* (c) cultivated in the liquid fermentation BJFA-MS medium or de Man, Rogosa, and Sharpe (MRS) broth, comparatively

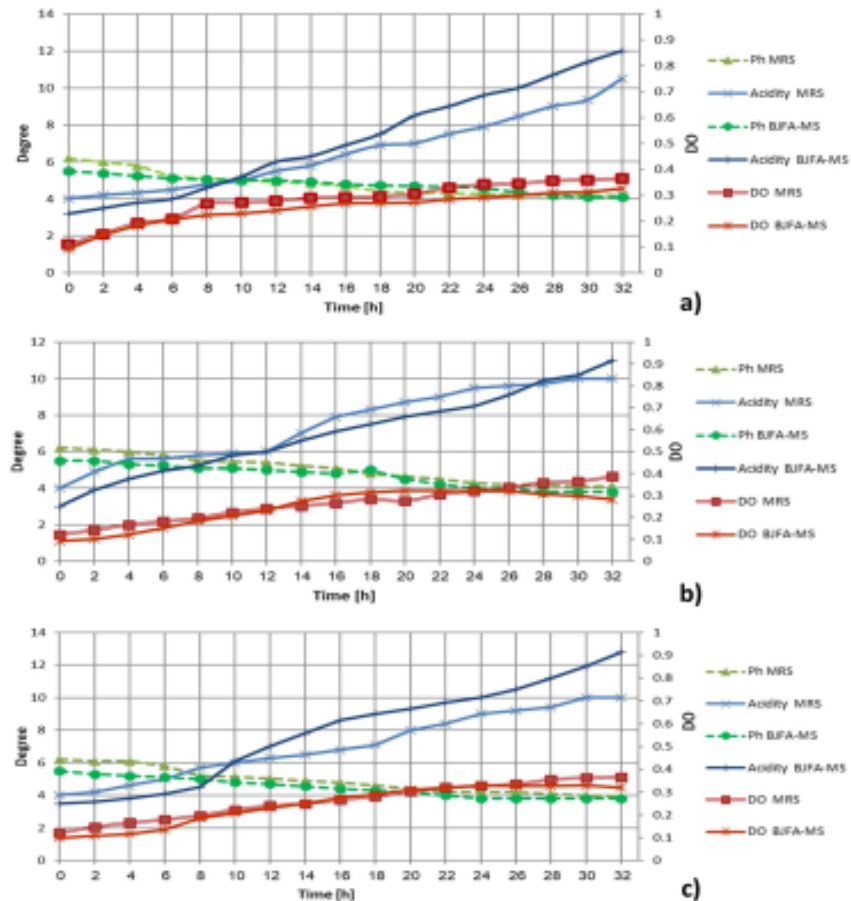


Table 3 Growth parameters of selected lactic strain cultivated in MRS and the BJFA-MS medium

Parameter ¹	μ_{\max} (h ⁻¹)		G (h)		Qac (g/g.h)		Productivity (g/L.h)	
	MRS ²	BJFA-MS ³	MRS	BJFA-MS	MRS	BJFA-MS	MRS	BJFA-MS
<i>L. plantarum</i>	0.014	0.015	49.5	46.2	1.03	1.7	2.1	1.75
<i>E. durans</i>	0.028	0.074	24.57	9.37	2.5	3.82	2.53	1.87
<i>L. fermentum</i>	0.034	0.05	20.39	13.9	1.85	4.05	2.1	1.8

1: Analysed parameters; μ_{\max} (h⁻¹)—maximum growth rate; G (h)—generation time; Qac (g/g.h)—Specific acid production rate in grams of acid formed per gram; Productivity (g/L.h)—the quantity of acid formed (g/L) per hour (h). 2: MRS is the abbreviation of de Man, Rogosa, and Sharpe broth. 3: BJFA-MS stands for the Beetroot Juice Fixed Additives-Manganese Sulfate

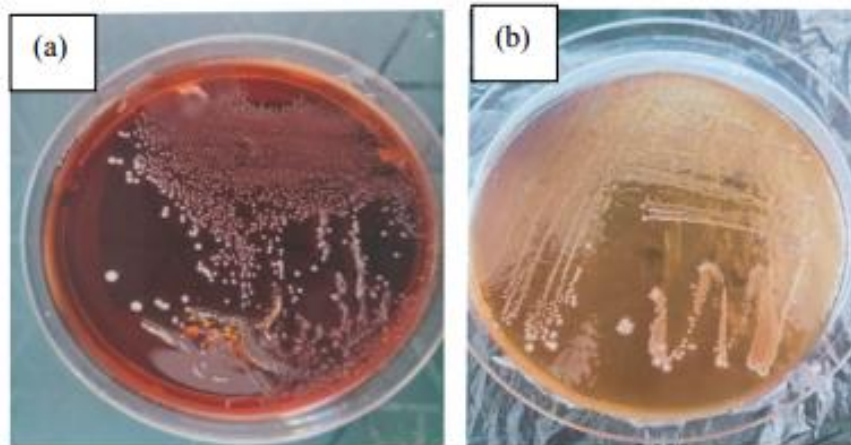
Agar plate growth

The nutritional requirements of lactic acid bacteria (LAB) are intricate, encompassing a spectrum of compounds such as carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, and vitamins (Hébert et al. 2009). Notably, the acquisition of nutrients, particularly amino acids, poses a significant challenge for LAB. To address this, chemically defined media have been devised to meet their specific nutritional needs. In the similar case of the study performed by (Ossa et al. 2010) where they underwent an Evaluation of cane molasses as a substrate for *Lactobacillus plantarum* growth, The findings of the study indicate that employing cane molasses as a substrate for the growth of *Lactobacillus* could present a cost-effective alternative to sourcing strains from abroad. This approach has the potential to lower expenses for consumers and offer advantages to the Colombian industry (Ossa et al. 2010). Furthermore, our experimental framework expanded to integrate specific chemical agents, including magnesium sulfate, manganese sulfate, sodium acetate, ammonium citrate, di-potassium phosphate, and zinc oxide, into the beetroot and additive mix. This comprehensive approach aimed to unravel the complex interactions among these components, shedding light on the feasibility of beetroot-derived medium to support the growth of LAB. Such findings carry implications for potentially replacing conventional MRS medium with beetroot-derived alternatives.

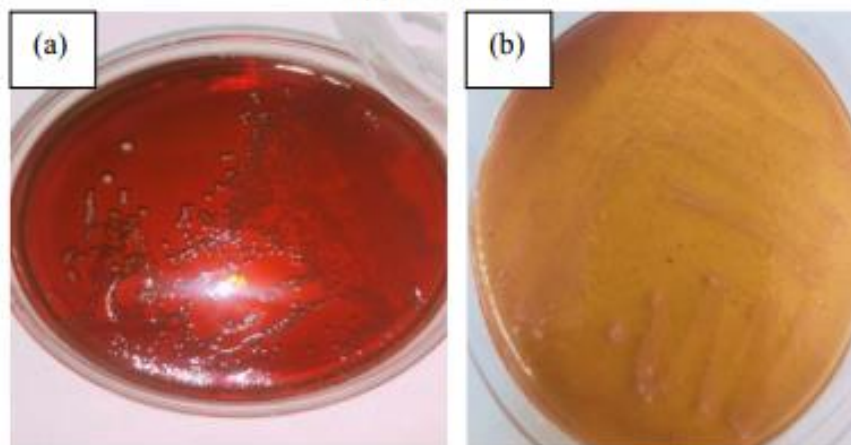
Additionally, among the various formulations tested, our study identified that the medium consisting of beetroot, additives mix, and manganese sulfate demonstrated superior performance for all three lactic acid bacteria strains examined. This formulation was subsequently selected for agar plate testing. Encouragingly, the results from the agar plate testing revealed promising outcomes when compared to the conventional MRS medium. Notably, the beetroot-based medium exhibited comparable macroscopic characteristics and, on average, yielded similar cell counts to those observed with the MRS medium. These findings underscore the potential of beetroot-derived alternatives in achieving outcomes akin to established media formulations, such as MRS medium, thereby offering avenues for cost-effective and sustainable solutions in lactic acid bacteria cultivation (Fig. 4).

Discussion

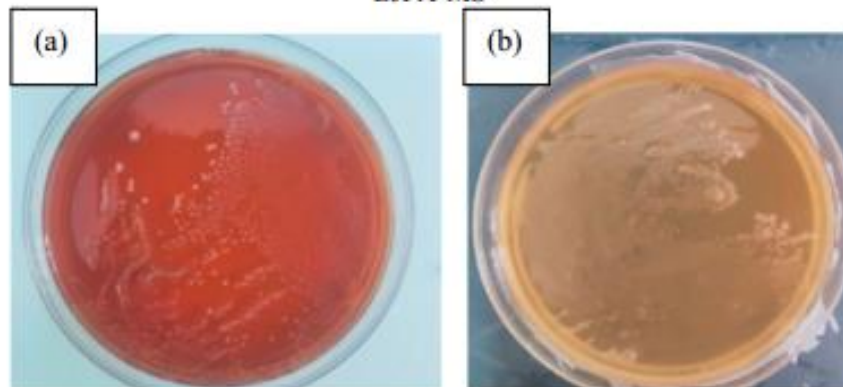
Regarding our findings, the first part was about phytochemical analysis which will be the stepping step to forecast the suitability of the medium to host lactic acid bacteria growth and proliferating. The pH value was 5.5, consistent with studies by Jafar et al. 2019 where pH values were reported between 5.4 and 6.5. It is noteworthy that pH measurement serves as an indicator of titratable acids in the product, influenced by both the inherent acids in the raw material and those generated during fermentation (Rakin et al. 2004; Xu et al. 2020). Titratable acidity serves as a dependable indicator of organic acid impact in foods. Our observation of a 13% titratable acidity in beet juice suggests a significant presence of organic acids. This finding aligns with prior studies by (Chen et al. 2017), (Lee et al. 2019), and (Johnson et al. 2022) indicating a Common titratable acidity range of 10 to 15 m.Eq in similar juice. The total sugar content in the beet-based medium, measured at 67 g/L, falls within the optimal sugar concentration range (50–70 g/L) known to support lactic acid bacteria growth and lactic acid production. Our analysis confirms a sufficient sugar concentration of 67 g/L, consistent with findings by Bujna et al. 2017 and Venegas al. (2019), reporting sugar concentrations around 60 to 70 g/L (Bujna et al. 2017; Venegas-Ortega et al. 2019). In contrast with the findings of Kazimierczak et al. 2014, research on dry matter content in beetroot juices our finding highlighted a notably lower value. Moreover, the extract and density values presented in our study exceed those in our previous investigation (Janiszewska-Turak et al. 2022), likely attributable to variations in vegetable maturity and experimental timing. The investigation into fermenting beetroot juice with lactic acid bacteria (LAB) represents a significant stride toward exploring alternative mediums for LAB growth and fermentation. The fermentation outcomes have shed light on the pivotal role of medium composition in shaping LAB growth dynamics. While beetroot juice alone exhibited limited growth, formulations enriched with additives demonstrated enhanced bacterial proliferation, emphasizing the significance of nutritional supplementation. Particularly noteworthy was the formulation featuring manganese sulfate (BJFA-MS), which emerged as a promising



Culture aspect of *Lactobacillus plantarum* in both (a) MRS medium and (b) BJFA-MS



Culture aspect of *Enterococcus durans* in both (a) MRS medium and (b) BJFA-MS



Culture aspect of *Lactobacillus fermentum* in both (a) MRS medium and (b) BJFA-MS

Fig. 4 Agar plating method for the medium BJFA-MS

contender to MRS medium, displaying comparable bacterial growth and macroscopic characteristics. This emphasizes the promise of semi-natural mediums derived from beetroot as cost-effective alternatives for LAB cultivation.

Further examination of kinetic parameters elucidated the performance of various mediums in supporting LAB growth. Although certain formulations showcased higher specific growth rates, a Focus on generation time highlighted the comparable efficacy of BJFA-MS, especially for strains like *L. fermentum* and *E. durans*. This underscores the potential of BJFA-MS as a viable alternative to traditional MRS medium, with profound implications for large-scale LAB cultivation. In comparing the growth kinetics of *Lactobacillus plantarum* across various media formulations to the reference MRS medium, distinct trends emerge. While beetroot alone yielded a notably slower growth rate with a higher maximum generation time, suggesting potential nutrient deficiencies, BJFA-PP and BJFA-MG showed comparable or slightly improved growth rates. Notably, BJFA-MS exhibited faster growth kinetics, indicating a more conducive environment for bacterial proliferation. These results are contrastable to the finding of Yoyok, Budi et al. (Yoyok, Budi, et al., 2010) where the maximum growth rate was found to be 0.17 h⁻¹ which is higher than our finding (Yoyok, Budi et al. 2010). this contrast can be explained as the conditions of culture may vary and the genera utilized have more performance regarding the enzymatic arsenal however it's a positive result since our findings are both in the positive spectrum.

Owing to the second case, about the kinetics of *Enterococcus durans* across different media formulations to the reference MRS medium, notable differences emerge. Beetroot alone demonstrated a markedly shorter maximum generation time of 0.091 compared to MRS's 50.22, suggesting faster growth. Similarly, across BJFA-MS, BJFA-MG, BJFA-ZO, and BJFA-PP formulations, *Enterococcus durans* displayed significantly shorter maximum generation times (0.073, 0.112, 0.109, and 0.031, respectively) compared to MRS. These findings collectively underscore the potential of beetroot-based media in promoting faster growth rates for *Enterococcus durans*, indicating their viability as alternatives to conventional MRS medium. Such outcomes hint at the possibility of harnessing beetroot-derived formulations to optimize microbial growth conditions, offering sustainable and efficient avenues for microbial cultivation. Finally, in the case of *Lactobacillus fermentum* growth kinetics across various media formulations against the MRS reference, notable differences emerge. Beetroot alone demonstrated a substantially reduced maximum generation time (15.75 h), suggesting its potential as a growth-enhancing medium. Conversely, the BJFA-ZO medium exhibited a significantly prolonged maximum generation time (77.01 h),

indicating potential growth inhibition with zinc oxide inclusion. While BJFA-SM and BJFA-MG showed comparable generation times to MRS, the BJFA-PP medium displayed a slightly shortened generation time (27.72 h), hinting at potassium phosphate's mild growth-promoting effect. These variations underscore the sensitivity of *Lactobacillus fermentum* to medium composition, emphasizing the need for nuanced formulation strategies to optimize growth conditions.

The Color-metrics analysis provided additional clarity regarding the transformative effects of LAB fermentation on beetroot juice. Substantial changes in color indexes, including an increase in the Yellow* value and a reduction in the Red* value, underscored the metabolic activity of LAB and its influence on pigment composition. These alterations, attributed to Lactic acid formation and pH reduction, highlight the potential of LAB-mediated fermentation in enhancing the nutritional and sensory properties of beetroot-based products. The agar plating results serve as a conclusive validation of BJFA-MS as a conducive growth medium for LAB showcasing comparable performance to MRS medium. This not only reaffirms the feasibility of beetroot-derived alternatives but also underscores their potential to offer cost-effective and sustainable solutions for LAB cultivation. Overall, the study opens new avenues for exploration in LAB cultivation and underscores the potential of beetroot-derived mediums in revolutionizing the field.

Conclusions

In the culmination of our study, delving into the fermentation of beetroot juice with lactic acid bacteria (LAB) unveils a promising frontier in alternative mediums for LAB growth and fermentation. Our findings from NEIRS reveal a pH value of 5.5, resonating with prior research and affirming the presence of essential titratable acids crucial for fermentation. Notably, the substantial titratable acidity of 13% in beet juice underscores its richness in organic acids, pivotal for fostering LAB proliferation. Moreover, the optimal sugar concentration within the beet-based medium accentuates its suitability as a favorable substrate for LAB growth, echoing its potential as a viable alternative to conventional mediums like MRS. The fermentation outcomes shed light on the pivotal role of medium composition in shaping LAB growth dynamics, with formulations enriched with additives displaying enhanced bacterial proliferation. The formulation featuring sulfate manganese (BJFA-SM) emerges as a standout contender to MRS medium, showcasing comparable bacterial growth and macroscopic characteristics. Further examination of kinetic parameters elucidates the performance of various mediums in supporting LAB

growth, with certain formulations exhibiting higher specific growth rates and shorter generation times, particularly noteworthy for strains like *L. fermentum* and *E. durans*. These results underscore the potential of semi-natural mediums derived from beetroot as cost-effective alternatives for LAB cultivation. Additional insights from color-metrics analysis highlight the transformative effects of LAB fermentation on beetroot juice, enhancing its nutritional and sensory properties. Conclusively, the agar plating results affirm BJFA-MS as a conducive growth medium for LAB, offering sustainable solutions and opening new avenues for exploration in microbial cultivation practices. Our study paves the way for further research endeavors aimed at optimizing sustainable microbial cultivation methods, thereby advancing food production and bolstering environmental stewardship.

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Author contributions Material preparation, data collection and analysis were performed by [Belhadj Oussama Kamel] [Hariri Ahmed], [Souafi Kheira] and [Missoum Ilham]. The first draft of the manuscript was written by [Belhadj Oussama kamel] and [Sahnouni Fatima]. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Authors' declaration The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Competing interests The authors declare no competing interests.

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Article 2.

Greener Cultivation: Replacing MRS Agar with Beetroot and soybean-formulated medium for Lactic Acid Bacteria Cultivation.



Greener Cultivation: Replacing Man Rogosa and Sharpe Agar with Beetroot and Soybean-Formulated Medium for Lactic Acid Bacteria Cultivation

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ABSTRACT

Lactic Acid Bacteria (LAB), widely used in food, health, and biotechnology, have driven interest in developing sustainable plant-based alternatives to conventional culture media. This study investigates alternatives to Man, Rogosa and Sharpe (MRS) medium for their cultivation. We evaluated the efficiency of Soybean-Beetroot Blend (SBB) in supporting the growth of *Lactobacillus plantarum*, *Enterococcus durans*, and *Lactobacillus fermentum*. Unlike prior studies, this research aims to optimize plant-derived media to address the specific nutritional needs of LAB bacteria. Initial trials showed that beetroot and soybean, when used separately, failed to sustain adequate bacterial growth, resulting in their elimination from further testing. Enriched media formulations - SBB and beetroot juice supplemented with Tween 80 (BJ-T80) - were subsequently assessed. Results demonstrated that SBB promoted the most efficient growth for *L. plantarum*, with a maximum specific growth rate (μ_{max}) of 0.248 h⁻¹ and a generation time of 2.79 h. In comparison, BJ-T80 exhibited a lower μ_{max} of 0.061 h⁻¹ and an extended generation time of 11 h, while the MRS medium achieved a μ_{max} of 0.154 h⁻¹. Acid production analysis revealed that SBB yielded a specific acid production rate (Q_{ac}) of 0.161 g/g/h and a productivity of 0.028 g/L/h. The BJ-T80 performed moderately better, with a Q_{ac} of 0.262 g/g/h and a productivity of 0.043 g/L/h, though MRS remained superior with a Q_{ac} of 1.588 g/g/h and a productivity of 2.100 g/L/h. These findings underscore the promise of beetroot-based media enhanced with plant-derived components as cost-effective and eco-friendly alternatives to conventional microbiological media.

Keywords: Soybean, Beetroot juice, Semi-natural media, Fermentation, *Lactobacillus plantarum*, *Enterococcus durans*.

Introduction

Sugars, organic acids, and minerals, which are abundant in fruits and vegetables, constitute key factors influencing properties such as sweetness, texture, and microbiological stability. As sustainability and natural alternatives gain increasing attention, the search for new approaches for lactic acid bacteria (LAB) cultivation has intensified, especially concerning the biovalorization of unexploited resources.¹ LAB, particularly species of the *Lactobacillus* genus, play a vital role in food fermentation, probiotics, and promoting overall health. However, they are known to be demanding microorganisms requiring specific growth conditions that are often lacking in conventional media. As highlighted by², the growth medium composition plays a crucial role in determining the growth rate, metabolic activity, and LAB viability. This recognition has driven increased interest in developing sustainable alternatives to conventional synthetic media. This study seeks to formulate a sustainable and complementary culture medium for lactic acid bacteria (LAB) by utilizing a combination of beetroot and soybean as nutritional substrates.

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To the best of our knowledge, there is currently no validated plant-based revalorized medium that has been confirmed as an effective substitute for the Man, Rogosa, and Sharpe (MRS) medium.⁴ This approach is motivated by the recognition of the nutritional value and bioactive compounds in these natural ingredients. Beetroot, known for its high content of sugars, vitamins and phytochemicals, effectively supports the growth and metabolic activity of LAB.⁵ Similarly, soybean is valued for its richness in high-quality proteins, essential amino acids, and bioactive components such as isoflavones, along with its lipid content, particularly polyunsaturated fatty acids.⁵ The study implemented a robust methodological framework, encompassing the selection of LAB strains, preparation of fermentation media, and evaluation of both phytochemical composition and microbial growth dynamics. In accordance with the methodologies adopted in earlier studies, an agar plating technique was employed to assess macroscopic features and enumerate viable cells. This work contributes to the development of this field by investigating the potential of plant substrates, including beetroot and soybean, for the formulation of environmentally friendly functional foods, probiotic products, and microbial carriers.

Materials and Methods

Harvesting and Drying Procedure of Vegetal Samples

Beet pulps and soybeans were obtained from local farms in Mascara, Algeria (GPS: 35.401864, 0.139919). They were then thoroughly cleaned to remove soil and debris. The leafy parts were carefully trimmed using a sterile scalpel, retaining only the necessary sections. The materials were then peeled, ground, and air-dried at room temperature (around 25°C) in a well-ventilated area. This natural dehydration process involves moisture removal through air circulation at ambient temperatures, without the use of artificial heat. After drying,

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the materials were ground into a fine powder using a mechanical grinder. They were then stored in airtight containers in a cool, dry place to preserve their stability.

Bacterial Strains and Culture Conditions

The three LAB strains used in this study were selected from a pool of 41 strains isolated from Khila, a traditional cheese from southwest Algeria. These strains were identified through 16S rDNA sequencing by LGC Genomics (Berlin, Germany), with sequence analysis performed using the NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST/>). This identification was part of a study titled "Sensory, microbiological and physico-chemical characterization of Khila, a traditional cheese made in the southwest of Algeria" by *. The used strains included *Lactobacillus plantarum* (from sheep's milk cheese), *Lactobacillus fermentum* (from cow's milk cheese), and *Enterococcus durans* (from goat's milk cheese). They were cultured at 30°C for 24 hours in MRS broth.

Phytochemical Analyses

Phytochemical analyses were conducted on 100 g samples of dried and finely ground beetroot and soybean powders using standard chemical assays. Nutritional components, including total sugars, protein, water, fat, fiber content, and humidity, were quantified following standard protocols. Calibration standards were prepared for accurate measurement and chemical reagents were handled according to standard procedures.¹ Titratable acidity and total sugar content in beet and soybean juices were determined using a pH meter (Model Metrohm pH-Meter 632 AG CH-9101 Herisan - Swiss made). Titratable acidity was measured by titration with a standardized 0.1 N NaOH solution of 98% purity to a final pH of 8.2, according to ISO 750:1998. Total sugar content was quantified using the phenol-sulfuric acid method: a phenol solution was prepared at a 5% (w/v) concentration (5 g of phenol per 100 mL of water) with a purity of 99%, involving a reaction with phenol and sulfuric acid, followed by spectrophotometric measurement (Model SPECORD® 50 PLUS BU-TTF from Analytik Jena company - Germany) at 490 nm. Both methods followed the protocols of ^{2,3} regarding the phenol-sulfuric acid method and ISO 12846:2012 for titratable acidity. All measurements were performed in triplicate.

High-Performance Liquid Chromatography Analysis

The soybean and beetroot medium contents were determined using HPLC analysis, with compounds identified according to Fluka and Sigma Aldrich standards, with a purity of 99.5%, using Shimadzu equipment (USA Manufacturing Inc, USA) consisting of two LC-20AD pumps, a CBM-20A controller, a SIL-20AC column oven, and an SPD-20AV UV/VIS spectrometer. Accurately weighed amounts of soybean and beetroot powder samples were placed in plastic test tubes. Then, 1 mL of methanol containing 1% ascorbic acid was added. The components were mixed well using a vortex device, and then incubated in an ultrasonic bath for 20 min at 20°C. After incubation, the samples were centrifuged at 3,920 × g. From each test tube, 1 mL of the extract was individually collected and re-centrifuged at 22,579 × g. Subsequently, 500 µL of each extract was transferred into HPLC vials for analysis. The concentration and retention time of each compound were expressed in ppm, following the protocol described by ¹⁰

Vegetal-formulated media Fermentation

A systematic series of experiments was conducted to evaluate the potential of soybean and beetroot media mixtures for lactic acid bacteria (LAB) cultivation. Initially, the individual effects of each component were assessed using standard protocols for media formulation and LAB cultivation, as described by ¹¹ and ¹². Subsequently, selected additives, including meat extract, yeast extract and chemical agents such as sodium acetate and ammonium citrate, at concentrations ranging from 1 g/L to 0.1 M, were incorporated into the media to evaluate their influence on LAB growth and metabolic activity.¹³ Next, we investigated the possible combinations of the two plant sources (soybean and beetroot), both individually and in combination, with supplementation, to compare their results with those of the standalone

media. This approach aimed to assess potential complementary interactions between the two plant sources, considering their distinct nutrient profiles and functional attributes, as highlighted by previous studies on plant-based media for microbial cultivation.¹⁴

Fermentation kinetics and analysis

A comparative study was conducted by fermenting selected lactic acid bacterial strains in MRS medium and formulated media derived from various tested plant sources. The fermentation experiments were performed under strictly controlled conditions, including temperature (37°C), and agitation (150 rpm).¹⁵ Key parameters, such as acidity, pH and optical density (OD), were monitored throughout the fermentation process. The fermentation kinetics were analyzed by assessing parameters such as the maximum specific growth rate (μ_{max}), generation time (G), acid production rate (Q_{ac}) and productivity (g/L/h), which represents the amount of acid produced per hour. ¹⁶ Samples were collected at regular intervals for comprehensive analysis, including pH measurements, optical density, and acid concentration determination. ¹⁷ These methods were adapted from established protocols for LAB fermentation. As well, measurements were performed in triplicate, ensuring consistency and reproducibility of the results. ¹⁸

Testing Growth on Agar Plates

Agar plates were prepared using fermentation-derived media supplemented with agar in order to solidify the medium. For inoculation, bacterial strains were adjusted to a standardized optical density (OD) of 0.1 at 600 nm (approximately 10⁸ CFU/mL), according to standard practice for ensuring consistent bacterial concentration. ²⁰ Following inoculation, the plates were incubated at 37°C for 24 to 48 hours under aerobic conditions to evaluate growth dynamics, macroscopic appearance, and colony characteristics, including size, shape, and color. Incubation was conducted at 37°C with a maintained relative humidity of 90%. ²⁰ Colony characteristics such as morphology (e.g., round, smooth, or rough), color (e.g., white, creamy), and texture were recorded to assess the growth performance of the strains across different media.

Statistical Analysis and Fermentation Parameters Calculation

To assess the effectiveness of the formulated medium on bacterial growth, a detailed analytical framework was implemented. Fermentation experiments were performed in triplicate. Statistical analyses were carried out to evaluate key fermentation parameters, including specific microbial growth rates. A one-way variance analysis (ANOVA) was employed to detect significant differences in parameters such as maximum specific growth rate (μ_{max}), generation time, acid production rate (Q_{ac}) and productivity across media, thereby highlighting statistically significant variations.

Results and Discussion

Phytochemical analysis

The chemical analyses of beetroot and soybean powders revealed distinct compositional characteristics, as summarized in Figure 1. Beetroot powder had a slightly acidic pH of 5.6, whereas soybean powder exhibited a pH of 6.2, consistent with findings by ²¹ and slightly higher than the typical 6.5-7.0 range reported by ²² Total sugar content was substantial in both powders, at 67% for beetroot and 70.2% for soybean, aligning with the generally high carbohydrate content of soybeans, though some studies indicate lower sugar levels. ²³ Water content was markedly higher in beetroot powder (87.6%) compared to soybean powder (63.1%), reflecting beetroot's higher moisture retention capability, as also noted by ²¹ and supported by other studies highlighting soybean's comparatively lower moisture retention. ²⁴ Protein content was significantly greater in soybean powder (41.67%) than beetroot powder (31.09%), aligning with soybean's known high protein composition. ²⁵ though some beetroot varieties have reported slightly higher protein contents. ^{24,26}

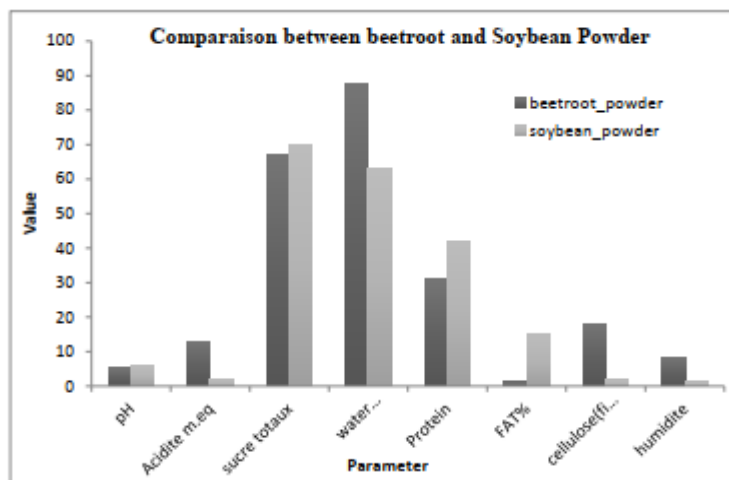


Figure 1: Chemical analysis of the soybean and beetroot powder

Similarly, fat content was substantially higher in soybean powder (14.89%) compared to beetroot powder (1.32%), consistent with soybeans' lipid-rich profile, whereas beetroot is recognized as having negligible fat content.⁴ Fiber content was notably greater in beetroot powder (18.32% cellulose) than soybean powder (2%), reflecting beetroot's higher dietary fiber content compared to the typically lower fiber levels in soybeans.²⁵ Lastly, moisture levels were higher in beetroot powder (8.45%) relative to soybean powder (1.35%), consistent with beetroot's higher water activity and soybean's lower moisture retention capacity (Figure 1).²⁶

The primary objective of this study was to assess the viability of lactic acid bacteria (LAB), specifically *Lactobacillus plantarum*, *Enterococcus durans* and *Lactobacillus fermentum*, in beetroot-based media and their formulations as sustainable alternatives to the conventional MRS medium. Previous studies exploring similar objectives frequently focused on fermentation or probiotic effects in food products like yogurt²⁵ or biscuits²⁶; in contrast, our research emphasized optimizing plant-based growth media. Prior investigations affirm the ability of LAB to grow successfully on plant-derived substrates, such as beetroot, with *Lactococcus lactis* and *Weissella cibaria* strains isolated from fermented beetroot products²⁵ and *Lactobacillus* species demonstrating viability in pasteurized beetroot juice without nutrient supplementation.²⁶ These findings support our observations, confirming beet juice as a suitable substrate for basic LAB cultivation, particularly for *L. plantarum* and *L. fermentum*. However, as highlighted by²⁷ beetroots alone might not offer optimal growth conditions for LAB, as demonstrated by the moderate growth of *Lactobacillus acidophilus* and *L. plantarum* in beetroot juice alone. Our findings corroborate this, indicating that soybean supplementation substantially enhances bacterial proliferation.

Our study demonstrates that a soybean-beetroot blend (SBB) supports bacterial growth comparable to the MRS medium, contrasting with previous studies where plant-based media underperformed traditional formulations.²⁷ Notably, growth parameters such as μ_{max} and generation time improved significantly in the SBB medium, underscoring its potential as a viable alternative to Man Rogosa Sharp. Although *L. plantarum* exhibited robust growth in this formulation, overall acid production and productivity remained inferior to MRS, which consistently promoted superior LAB growth.^{14,28} This discrepancy underscores the necessity for further optimization of the

soybean-beetroot medium to enhance LAB proliferation and acid production effectively.

Fermentation

This experiment involved the cultivation of three lactic acid bacteria, namely *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Enterococcus durans*, in soybean and beetroot juice individually, as well as in various synergistic formulations. The initial inoculum of all lactic acid bacteria in fermented juices was calibrated at 10^7 logs CFU/mL with optical density serving as an indicator of bacterial growth and medium consumption (Figures 2, 3, 4). Multiple contrasts were observed between soybean juice and beetroot juice, whether alone or with the mixed formulation.

Beetroot juice alone exhibited the lowest growth among the three lactic acid strains throughout the fermentation period. This may be attributed to its limited nutritional value and the high nutritional requirements of lactic acid bacteria, which are known to be fastidious in their trophic requirements. Slightly improved bacterial growth was observed when beetroot juice was supplemented with meat extract and yeast extract. These results are similar to those described by²⁹. They exhibited observable growth in *L. plantarum*, with a progressive increase in the number of viable cells over time when beetroot juice was used as the fermentation substrate. This is further supported by³⁰ who successfully fermented beetroot-based media using lactic acid bacteria, demonstrating that adequate nutritional supplementation is essential for the minimal growth requirements of these microorganisms.³⁰

Kinetic Findings

Although MRS medium remains the gold standard for LAB cultivation, increasing interest in sustainable alternatives has driven the development of plant-based media. This study highlights the effectiveness of beetroot-based formulations in promoting LAB growth. Notably, beetroot juice alone resulted in the highest maximum specific growth rate (μ_{max}) for *L. plantarum* (0.222 hr⁻¹), surpassing both MRS (0.154 hr⁻¹) and soybean-based media. This can be attributed to the abundance of bioactive compounds present in beetroot, such as betalains and flavonoids,³¹ supporting findings that emphasize the potential of plant-derived media.²³

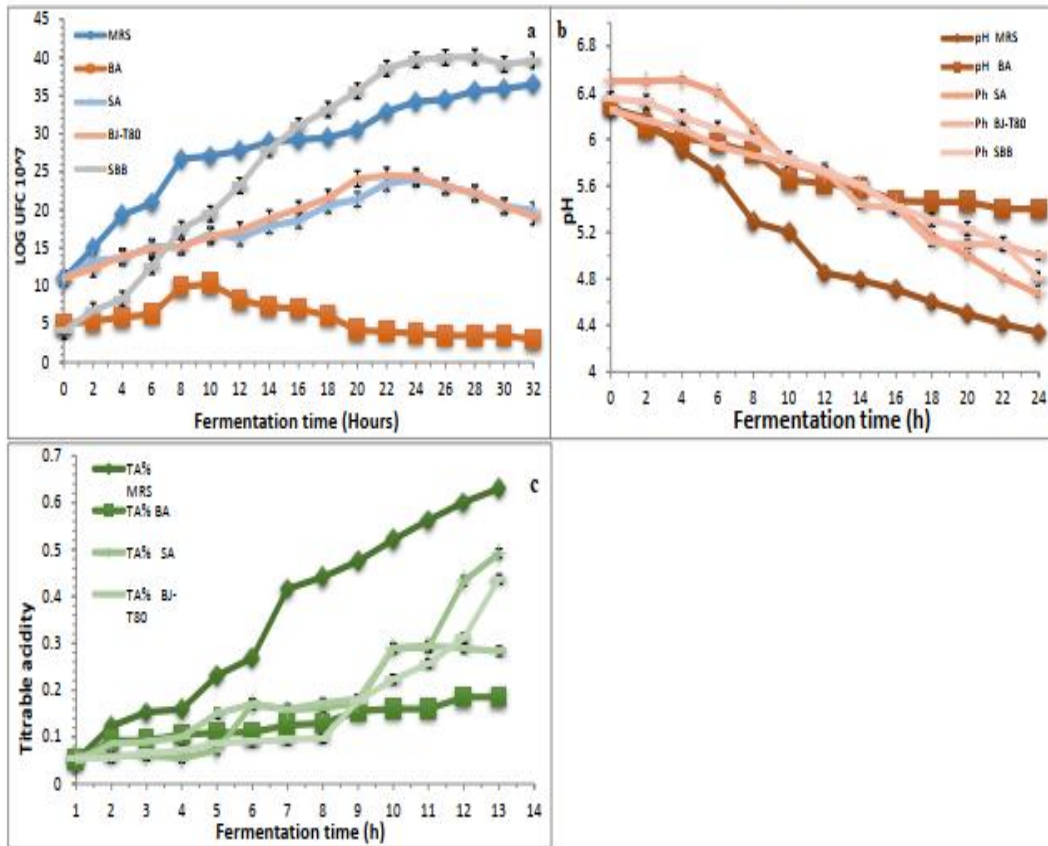


Figure 2: Log CFU ml⁻¹ pH and titratable acidity of *Lactobacillus plantarum* strains in different growth media: (a) Viable LAB bacteria count; (b) pH; (c) Total titratable. All treatments were carried out in triplicate.

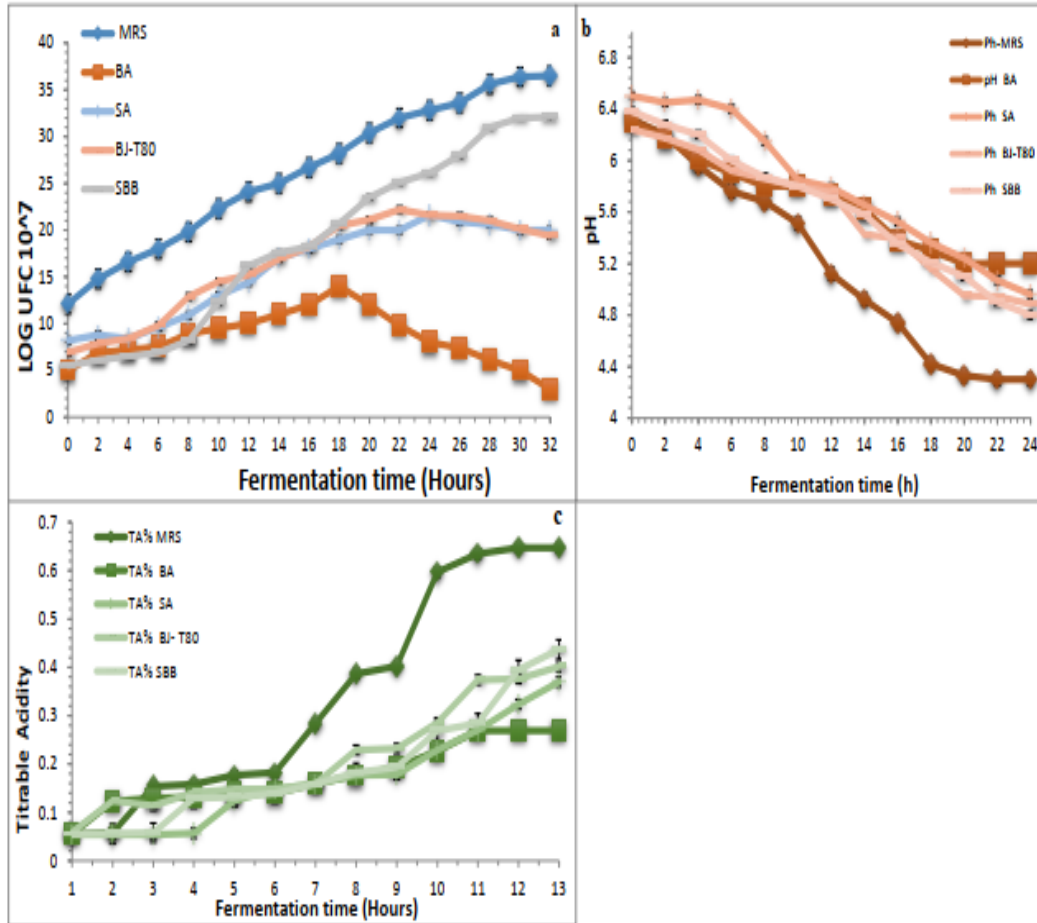


Figure 4: Log CFU ml⁻¹, pH and acidity of *Lactobacillus Fermentum* strains in different growth media: (a) Viable LAB bacteria count; (b) pH; (c) Total titratable acidity. Experimental data were fitted to the logistic model. All treatments were performed in triplicate

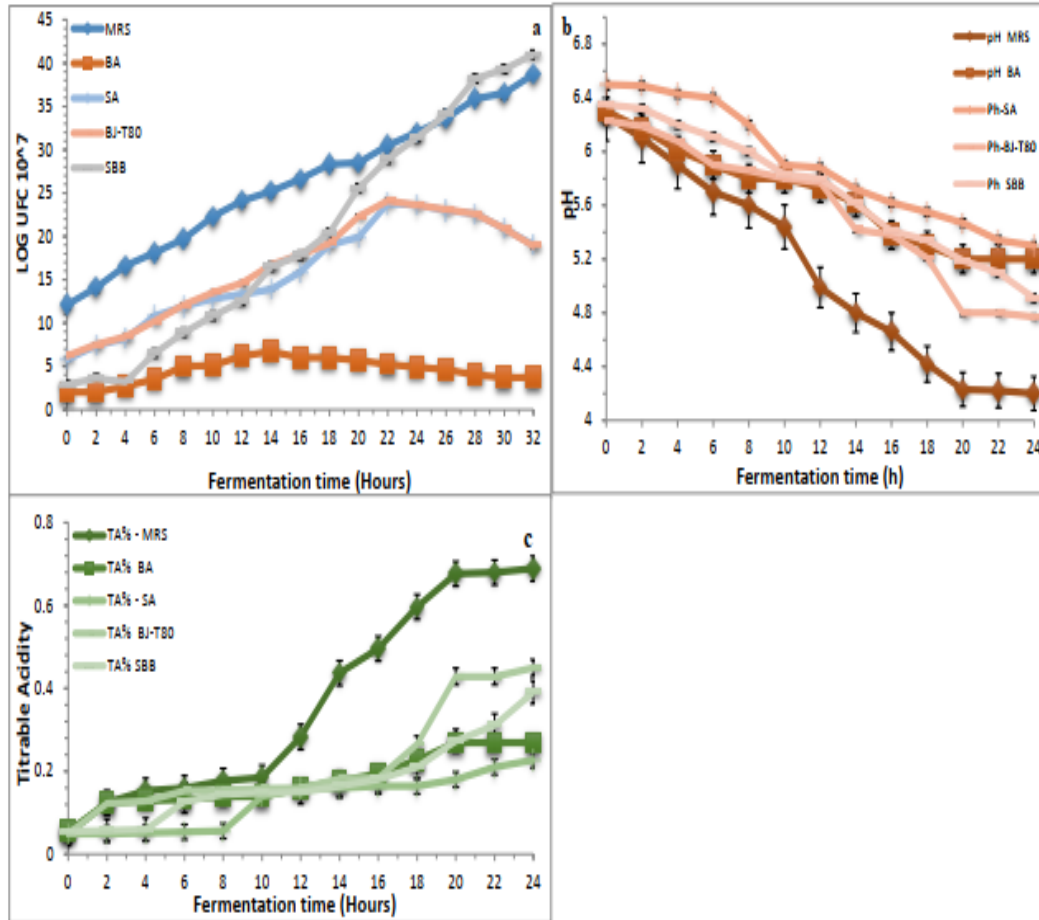


Figure 3: Log CFU ml⁻¹, pH and acidity of *Enterococcus durans* strains in different growth media: (a) Viable LAB bacteria count; (b) pH; (c) Total titratable. Experimental data were fitted to the logistic model. All treatments were performed in triplicate.

Table 1 summarizes kinetic parameters, showing that *L. plantarum* exhibited the fastest generation time in beetroot juice alone (3.122 h), whereas the soybean-beetroot blend (SBB) formulation emerged as the most efficient medium for *E. durans* (2.037 h), highlighting the advantage of targeted nutrient supplementation.⁴¹ Although BJ-T80 exhibited slower growth, it nonetheless supported LAB proliferation, indicating potential for further medium optimization. Specific growth parameters (see Figure 5) also confirmed the superior performance of the SBB formulation. For *L. plantarum*, SBB showed a μ_{max} of 0.248 h⁻¹ and a generation time of 2.79 hours, while BJ-T80 had lower growth and higher generation time (μ_{max} of 0.061 h⁻¹ and

11 hours, respectively), with MRS medium intermediate between these values (μ_{max} of 0.154 h⁻¹ and generation time of 4.5 hours). For *E. durans*, SBB markedly outperformed BJ-T80 (μ_{max} of 0.340 h⁻¹ and generation time of 2.037 h), whereas MRS underperformed comparatively (μ_{max} of 0.081 h⁻¹). Similarly, for *L. fermentum*, SBB again excelled (μ_{max} of 0.200 h⁻¹), outperforming BJ-T80 (μ_{max} of 0.137 h⁻¹) and significantly surpassing MRS medium, which showed the lowest efficiency (μ_{max} of 0.100 h⁻¹). These results align with³⁶ who demonstrated that *Lactobacillus acidophilus* effectively fermented a soybean-based beverage, achieving a viable cell count of 4.66 log CFU/g.

Table 1: Growth parameters of the selected lactic strain cultivated in MRS and the medium

Parameter	Qac (g/g.h)			Productivity (g/L.h)		
	<i>L. plantarum</i>	<i>E. durans</i>	<i>L. fermentum</i>	<i>L. plantarum</i>	<i>E. durans</i>	<i>L. fermentum</i>
MRS ¹	1.588 ± 0.6	4.055 ± 0.6	1.852 ± 0.6	2.100 ± 0.8	2.531 ± 0.8	2.100 ± 0.8
BA ²	0.310 ± 0.6	0.290 ± 0.6	0.320 ± 0.6	0.155 ± 0.8	0.145 ± 0.8	0.160 ± 0.8
SA ³	0.316 ± 0.6	0.336 ± 0.6	0.327 ± 0.6	0.030 ± 0.8	0.025 ± 0.8	0.027 ± 0.8
BJ-T80 ⁴	0.262 ± 0.6	0.536 ± 0.6	0.430 ± 0.6	0.043 ± 0.8	0.061 ± 0.8	0.062 ± 0.8
SBB ⁵	0.161 ± 0.6	0.701 ± 0.6	0.530 ± 0.6	0.028 ± 0.8	0.029 ± 0.8	0.028 ± 0.8

¹: Man, Rogosa, and Sharpe; ²: Beetroot alone; ³: Soybean alone; ⁴: SBB: Stands for soybean-beetroot blend; ⁵: Beetroot juice without Tween 80. The findings are shown as mean ± standard deviation (SD).

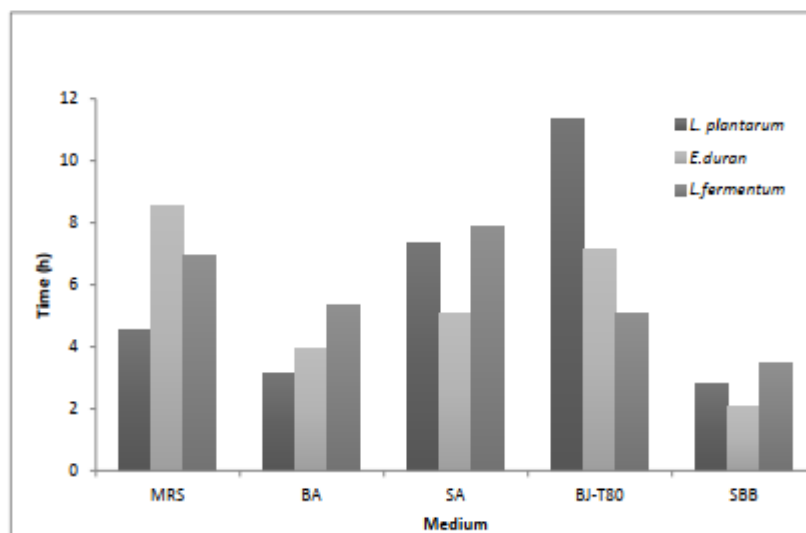


Figure 5: Results of kinetic fermentation of different beetroots with lactic acid bacteria, including MRS (Man, Rogosa and Sharpe), BA (beet alone), SA (soybean alone), SBB (soybean-beetroot mixture) and BJ-T80 (beet juice without Tween 80).

Furthermore, MRS medium consistently exhibited superior performance, achieving the highest Qac (1.588 g/g-h for *L. plantarum*) and productivity (2.10 g/L-h), confirming its established efficiency in LAB cultivation.⁴¹ Conversely, beetroot alone (BA) and soybean alone (SA) showed significantly lower Qac values (0.310 and 0.316 g/g-h, respectively) and reduced productivity, highlighting their limited capability to support high acid production. Introducing additives into BJ-T80 slightly improved performance, particularly for *L. plantarum* and *E. durans*, although these

enhancements still did not match the performance of MRS. This finding aligns with⁴⁴ who observed that additives could enhance LAB growth but typically yield inferior results compared to traditional media. SBB medium demonstrated better outcomes, particularly for *E. durans* (Qac of 0.701 g/g-h, productivity of 0.029 g/L-h), underscoring the potential of plant-based media when supplemented with appropriate nutrients. Specifically, for *L. plantarum*, SBB yielded moderate parameters (Qac of 0.161 g/g-h, productivity of 0.028 g/L-h), while BJ-T80 showed improved values (Qac of 0.262 g/g-h, productivity of 0.043 g/L-h).

however, MRS still outperformed both. For *E. durans*, BJ-T80 exhibited lower Qac (0.536 g/g-h) but higher productivity (0.061 g/L-h), whereas MRS had the highest performance (Qac of 4.055 g/g-h, productivity of 2.531 g/L-h). Likewise, for *L. fermentum*, SBB recorded a Qac of 0.530 g/g-h and productivity of 0.028 g/L-h, whereas BJ-T80 demonstrated lower Qac (0.430 g/g-h) but higher productivity (0.062 g/L-h), with MRS remaining superior (Qac of 1.852 g/g-h, productivity of 2.100 g/L-h). These findings highlight the necessity for further optimization of plant-based media to achieve improved acid production and productivity, challenging the prevailing view that MRS universally provides optimal conditions.⁴⁵

High-Performance Liquid Chromatography










Figure 6 presents the growth parameters of selected LAB strains cultivated in MRS and SBB media, highlighting the correlation between key bioactive compounds and their concentrations. Calibration curves for benzoic acid, gallic acid, quercetin, and trans-cinnamic acid showed strong correlations (r^2 values ranging from 0.99962 to 0.99998), confirming accurate quantification of these compounds. Figure 7 displays the HPLC chromatogram, revealing four significant peaks corresponding to these compounds. Quercetin exhibited the highest concentration (47.629 ppm) with a retention time of 47.6 minutes, indicating its predominant role in LAB growth regulation.⁴⁶ Gallic acid and benzoic acid were present in lower concentrations (5.912 ppm and 76.313 ppm, respectively), yet still demonstrated strong correlations, suggesting their involvement in modulating LAB metabolism. Trans-

cinnamic acid, detected at 7.207 ppm, aligns with its recognized influence on bacterial growth.⁴⁷ The HPLC analysis emphasizes distinct roles of these bioactive compounds in LAB metabolic activities. Benzoic acid, though inhibitory at elevated concentrations, was present at LAB-tolerable levels, maintaining medium stability without negatively impacting growth.⁴⁸ Gallic acid functions as a protective agent, enhancing bacterial resilience under oxidative stress conditions and promoting survival during metabolism.⁴⁹ Similarly, quercetin aids bacterial survival in oxidative environments, indirectly supporting growth.⁴⁹ Trans-cinnamic acid stimulates adaptive metabolic responses at low concentrations, potentially improving LAB metabolic flexibility, although careful control of concentration is necessary to prevent inhibition.⁴⁸ These findings underscore the critical role of phenolic acids and flavonoids in optimized plant-based media formulations. While the significant presence of these compounds contrasts with reports of their inhibitory effects on specific LAB strains, the data indicates that controlled concentrations and compositions in SBB media can beneficially modulate LAB growth and metabolic activity.

Agar plate growth

The agar plate culture of the lactic acid bacteria displayed clearly distinguishable colonies, evenly distributed and easily enumerable. Consistent colony morphology and uniform sizes indicate bacterial purity and optimal growth conditions. The colonies were mostly white or milky white, with a round shape, neat edges, and a moist, smooth surface. (Table 2).

Table 2: Growth result of formulated medium which showed lactic acid bacterial growth.

Medium	Cultural aspect		
	<i>L. plantarum</i>	<i>E. durans</i>	<i>L. Fermentum</i>
MRS ¹			
BJ-T80 ²			
SBB ³			

¹MRS: Man, Rogosa and Sharpe; ²BJ-T80: Beetroot juice auditioned with Tween 80; ³SBB: Soybean beetroot blend.

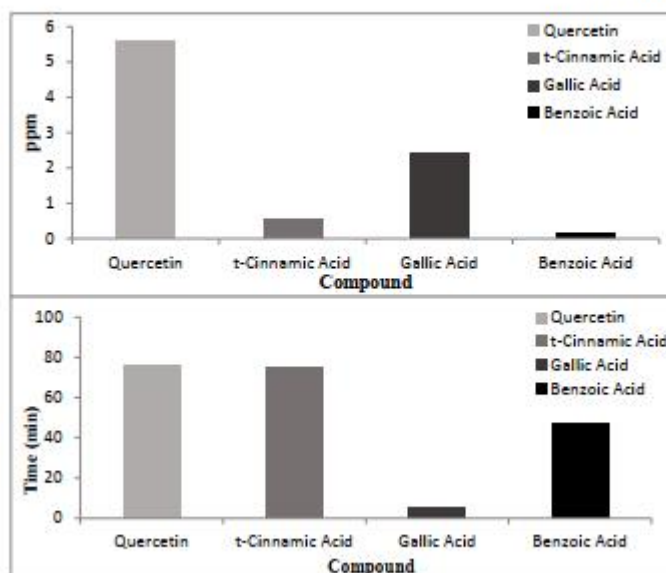


Figure 6: Compounds present in the SBB medium: (a) retention time; (b) quantities.

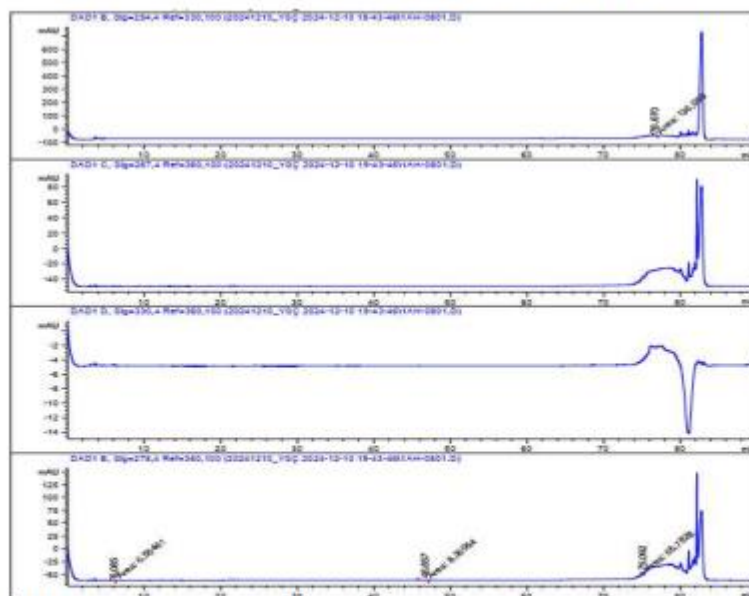


Figure 7: The HPLC chromatograms of the soybean beetroot blend (SBB) medium : (1) Quercetin; (2) t-Cinnamic Acid; (3) Gallic Acid; (4) Benzoic Acid.

Although MRS medium consistently supports the best performance across all LAB strains, our findings demonstrate that our plant-based media, composed of beetroot and soybean, especially when supplemented with appropriate additives, have the potential to substitute or supplement conventional media like MRS (Table 2).^{18,44} Further optimization of these plant-based formulations could lead to more sustainable, cost-effective, and eco-friendly alternatives for LAB cultivation in various biotechnological applications.^{45,46} These results underscore the clear advantages of MRS medium for acid production and productivity. However, the SBB medium was found to be the most favorable for bacterial growth, followed by BI-T50, demonstrating that soybean extract could replace Tween 80 while still enhancing LAB growth. This suggests that with further optimization, plant-based formulations could serve as cost-effective and sustainable alternatives to MRS for LAB growth.

Conclusion

This study successfully demonstrated the feasibility of using a soybean and beetroot-based medium to support the growth of lactic acid bacteria (LAB). The results highlighted the potential of plant-based semi-natural media as cost-effective alternatives to conventional MRS media for cultivating *Lactobacillus plantarum*, *Enterococcus durans*, and *Lactobacillus fermentum*. The HPLC analysis identified benzoic acid, gallic acid, quercetin, and trans-cinnamic acid in the beetroot and soybean medium, which supporting LAB growth and metabolic activity. These compounds enhance oxidative stress resilience, metabolic adaptability as well as medium stability at appropriate concentrations. Our study revealed that the SBB medium was comparable to its second formulation, containing Tween 80, and could provide the same effect. Overall, our findings provide compelling numerical and practical evidence that the development and optimization of our soybean-beetroot-based medium offers a sustainable and efficient alternative for LAB cultivation. While these novel media have demonstrated their ability to support bacterial growth and lactic acid production, future investigations should determine their suitability either as proliferation media or as media dedicated to metabolite production.

Conflict of interest

The author's declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Annexes

1-Spectrophotometer Model : SPECORD® 50 PLUS
Manufacturer : Analytik Jena
Country : Germany



2-pH meter

Model: Metrohm 632

ID-number: 011650

Country: Switzerland

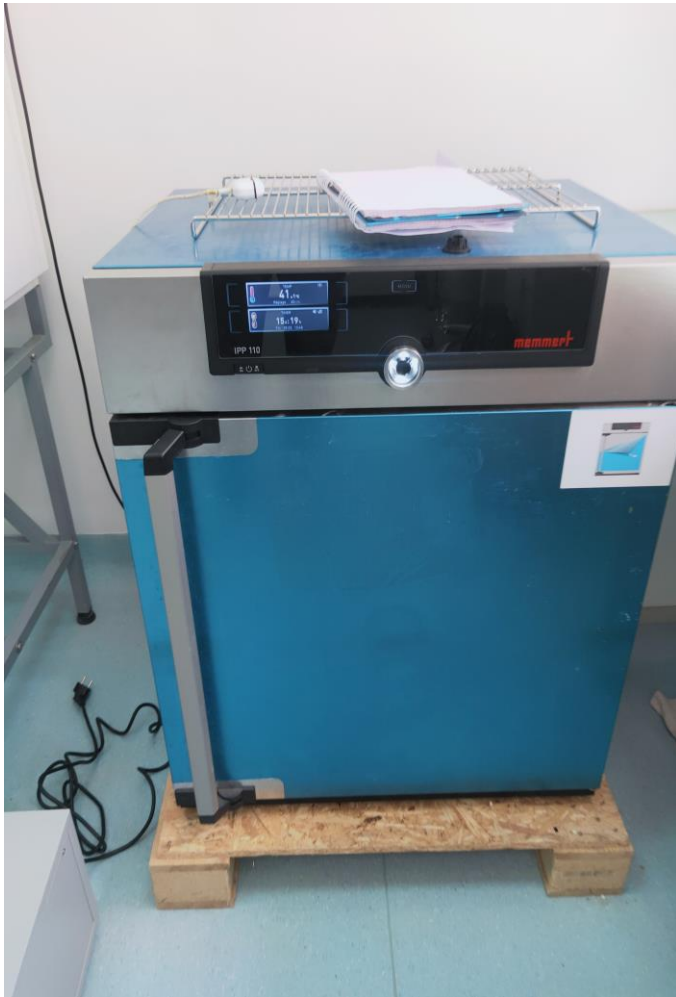


3-Incubateur

Model: IPP110eco

Manufacturer: Memmert

Country: Germany



4- Anaerobic jar



